THE CHLORAMPHENICOL BINDING SITE OF MAMMALIAN MITOCHONDRIAL RIBOSOMES

BY

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A DISSERTATION PRESENTED TO THE GRADUATE COUNCIL
OF THE UNIVERSITY OF FLORIDA IN
PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1982

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ACKNOWLEDGEMENTS

I would like to gratefully acknowledge Robert Sheehan, Robert Cheatham, and Jane Edwards, for their technical support, especially in the area of ribosome preparation. In addition, I would like to thank Jane Edwards for her technical expertise in performing two-dimensional PAGE. I would like to acknowledge Mary Conde for providing information about the irreversible inactivation of mitochondrial ribosomes by iodoamphenicol and for the preparation of the bacterial ribosomes.

I acknowledge the good times and criticisms provided by Gretchen Schieber and Sal Pietromonaco, during my graduate career.

Nancy Denslow receives a special acknowledgement for donating the tRNA substrates and also as an influential scientist in shaping my understanding of mitochondrial ribosomes.

Tom O'Brien receives a most grateful acknowledgement. He is truly the most observant student of the empirical sciences and human nature that I know. His guidance has been, above all, a remarkable experience for me.

I would like to thank my wife, Jean, and my parents for their patience.

Finally, I would like to thank Donna VanPuymbrouck, who provided the secretarial skills to bring about this manuscript.

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ABBREVIATIONS

Chloramphenicol and Chloramphenicol Analogs

CAP	Chloramphenicol
ACAP	p-Amino-chloramphenicol
FAP	p-Azidobenzoamido-chloramphenicol
HACAP	p-Hydroxylamino-chloramphenicol
IAP	Iodoamphenicol
PAF	Chloramphenicol-p-azidobenzoate
PAP	p-Azido-chloramphenicol

Other Abbreviations Used

AA-tRNA	Amino acyl transfer RNA
% C	Bisacrylamide concentration divided by the acrylamide plus the bisacrylamide concentration x 100
CPM	Counts per minute
f-Met-tRNA	Formyl-methionyl transfer RNA
H-bond	Hydrogen bond
IAM	Iodoacetamide
LSC	Liquid scintillation counting
K'_d	Apparent disociation constant
2ME	2-Mercaptoethanol
mRNA	Messenger RNA

N-Acetyl-AA-tRNA	N-Acetyl-amino acyl transfer RNA
NMR	Nuclear magnetic resonance
1-D	One-dimensional
PAGE	Polyacrylamide gel electrophoresis
PTase	Peptidyl transferase
rRNA	Ribosomal RNA
S	Svedberg unit
SDS	Sodium dodecyl sulfate
% T	Total acrylamide plus the bisacrylamide concentration x 100
2-D	Two-dimensional
TLC	Thin layer chromatography
tRNA	Transfer RNA

Abstract of Dissertation Presented to
the Graduate Council of the University of Florida
in Partial Fulfillment of the Requirements for
the Degree of Doctor of Philosophy

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December, 1982

Chairman: Thomas W. O'Brien
Major Department: Biochemistry and Molecular Biology

Chloramphenicol (CAP) is an antibiotic of therapeutic importance which inhibits protein synthesis in mammalian mitochondria as well as in its intended target, bacteria. CAP binds with high affinity to a site on the large subunits of ribosomes from bacteria and mitochondria. These ribosomes resemble each other in a primary functional manner, in that each is involved in a complete translational system. Yet, these ribosomes are very different in their physical-chemical properties. In spite of differences in the properties of these ribosomes, the binding of CAP to the Peptidyl Transferase (PTase) Center and the conservation of

protein synthesis suggests that the fundamental functional groups required for protein synthesis and CAP binding have been maintained on both kinds of ribosomes.

CAP is a relatively small antibiotic with a well defined bacterial site of action. Previous studies with bacterial systems have implied that CAP could be divided into three distinct regions that are required for activity. In the present study, CAP was chemically modified in two of these regions, the dichloroacetate chain and the para-nitrophenyl ring, and the resultant analogs were tested for the inhibition of the PTase activities of mitochondrial and bacterial ribosomes. In this manner, the CAP binding site from these ribosomes was examined to determine if any of the analogs could discriminate between the ribosomes and to identify features of the CAP molecule which were important for binding to this site. The resultant data provided evidence that the para-nitro moiety was hydrogen bonded to some component of the ribosome at the CAP binding site and that a limited volume or bulk could be accommodated in the position of the dichloroacetate chain. Some of the CAP analogs contained electrophilic groups, so that these CAP analogs could act as "affinity probes" for proteins of the CAP binding site. Protein L2 of the mitochondrial ribosome was identified, through the use of iodoamphenicol as a CAP affinity probe, as being within the domain of the PTase Center and CAP binding site of these ribosomes.

Using the functional and structural data obtained, models are presented, detailing the CAP binding site of mammalian mitochondrial ribosomes.

CHAPTER I
INTRODUCTION

Properties of Mammalian Mitochondrial Ribosomes

Protein synthesis occurs on a macromolecular complex of protein and ribosomal RNA (rRNA) determined to be an important entity of the cell more than 20 years ago. The "ribosome" became the word coined to represent this ribonucleoprotein complex (1). Ribosomes were found not only in bacteria and within the cytoplasm of the eukaryotic cell, but, in addition, during the mid-1960's were found by O'Brien and Kalf to inhabit the subcellular organelle, the mitochondria, of mammals (2, 3). These mammalian mitochondrial ribosomes are a component of a complete translation system within mitochondria, where they use mitochondrial mRNA's and tRNA's to synthesize about a dozen different proteins most of which belong to the electron transport system and ultimately reside in the inner mitochondrial membrane (4, 5). The protein synthesizing capability of the mitochondrial ribosome is required for the eukaryotic cell to maintain the integrity of its energy transducing system.

The mammalian mitochondrial ribosomes resemble prokaryotic ribosomes and eukaryotic cytoplasmic ribosomes in their basic organization, in that each consist of a large number of proteins and rRNA partitioned into two subunits which house the individual functions necessary to bring about protein synthesis. The major functional site on the ribosome is the Peptidyl Transferase (PTase) Center which is located on the larger of the two subunits of each of the ribosome types . The PTase Center is also the prime site for antibiotic action and details of its function and the binding of antibiotics will be discussed in later sections (6).

Size of the Ribosome

The mammalian mitochondrial ribosome was found to have a sedimentation coefficient of 55S which is much smaller than the values observed for prokaryotic ribosomes and eukaryotic cytoplasmic ribosomes with sedimentation coefficients of about 70S and 80S, respectively (Table I) (7, 8). Initially it was thought that mitochondrial ribosomes were smaller than their counterparts, based on the sedimentation coefficients observed (9). Even some investigators suggested that the 55S ribosome was a degradation artifact of a larger ribosome. Determination of a molecular weight of 2.8 million for the mammalian mitochondrial ribosome, which is slightly larger than the molecular weight of 2.7 million observed for bacterial ribosomes, helped to end the "small or degraded ribosome"

TABLE I

Properties of Ribosomes

Ribosome Type	S	MW*	Buoyant Density	rRNA MW*	Proteins
Mammalian Mitochondrial	55S	2.8	1.44	0.35 0.54	80-90
E. coli	70S	2.7	1.64	0.56 1.10	54
Mammalian Cytoplasmic	80S	4.5	1.58	0.7 1.5	70-80

* (MW x 10^{-6} Daltons)

notion connotated by a sedimentation coefficient of 55S (7, 10). The rather low sedimentation coefficient, 55S, is the result of a low buoyant density of about 1.44 g/ml for the mitochondrial ribosome as compared to about 1.64 g/ml for bacterial ribosomes (Table I) (7, 8, 11).

RNA Content

The low buoyant density and consequently the low sedimentation coefficient reflect not a smaller size of the mitochondrial ribosome when compared with the bacterial ribosome, but they reflect an alteration in the RNA and protein content of the mitochondrial ribosome. The mammalian mitochondrial ribosome has two RNA species which sediment at 16S and 12S, and have molecular weight of 0.54 million and 0.35 million respectively (7, 8). These ribosomal RNA's are about one-half the size of the comparable rRNA's from E. coli ribosomes which have molecular weights of 1.1 million and 0.56 million, respectively, and sediment at 23S and 16S, respectively (12).

Protein Content

The low buoyant density, the low sedimentation coefficient, and the smaller rRNA's indicate that mitochondrial ribosomes must have the bulk of their total mass contributed to by protein when compared to the bacterial ribosome. This is, in fact, the case. The mammalian mitochondrial ribosome has about 90 proteins which total to 1.81 million daltons and which comprise about

two-third's of the ribosomal mass (13). In comparison, the bacterial ribosome has 54 proteins of 0.98 million daltons and this amounts to slightly more than one-third of the bacterial ribosomal mass (14). The excess protein content of mammalian mitochondrial ribosomes, when compared with bacterial ribosomes, leads to interesting questions as to what functional and structural roles these proteins may have in addition to the primary protein synthetic activity of the ribosome.

Some of these proteins may have replaced the rRNA in the mitochondrial ribosome, yet others must be performing the same functional tasks as the analogous proteins from the bacterial ribosome. The mammalian mitochondrial ribosome with 52 proteins in its large subunit contains almost as many proteins as the entire bacterial ribosome, which has 54 proteins. Several of these proteins are certainly involved in the peptidyl transferase activity and some of these must be intimately associated with the chloramphenicol (CAP) binding site. The present study examines these functionally important proteins and resolves questions about the nature of the chloramphenicol binding site and the Peptidyl Transferase Center.

The Peptidyl Transferase Center

The function of the ribosome is to synthesize proteins by catalyzing the formation of peptide bonds between amino acids at the primary functional site on the large ribosomal subunit in the region termed the Peptidyl Transferase

Center. The catalytic activity at this site is called peptidyl transferase.

Why has such a complex organelle evolved to house this function and to bring about protein biosynthesis? A proteolytic-type enzyme could synthesize peptide bonds, but the essential features for the fidelity of protein synthesis would be lost. The ribosome provides an essential platform for the substrates and factors of protein synthesis to come together in an orderly fashion for the faithful translation of the genetic material, which apparently would not occur in a less complex system.

Substrates: Amino Acyl-tRNA and Peptidyl-tRNA Binding Sites

The Peptidyl Transferase Center is characterized by two adjacent binding sites for the substrates of peptide bond formation. Operationally these have been defined as the A site, for amino acyl-tRNA binding, and P site, for peptidyl-tRNA binding (6, 15). The P site accommodates the growing peptide chain attached to tRNA or the initial f-Met-tRNA. The A site is where the incoming amino acyl-tRNA binds prior to peptide bond formation. After transfer of the peptide chain, from the P site, to the amino moiety of the amino acyl-tRNA at the A site and removal of the uncharged tRNA from the P site, the new peptidyl-tRNA "translocates" to the vacated P site, opening the A site for a new amino acyl-tRNA and a new round of peptide bond synthesis (16). This process repeats until a protein has been synthesized.

Catalytic Activity

Molecularly, protein synthesis is not well understood. What enzymatic functions are required for peptide bond formation and protein biosynthesis? In recent years, two models have been proposed to explain the catalysis of peptide bond formation, i.e. peptidyl transferase. In the first model, peptide bond synthesis occurs by essentially the reversal of proteolysis, as if an acid protease such as chymotrypsin was performing the back reaction (17, 18, 19). This model invokes an active serine and histidine at the PTase Center (17). The major consequence of this model is the "acyl-ribosome" intermediate which would be generated by the serine attack on peptidyl-tRNA (17). No such intermediate has been generated by classical enzymological techniques, however (17). Another model, the "template" model, simply uses a ribosome as a template to bind the substrates and bring them into close proximity and the correct orientation for the chemical attack of the amino moiety of amino acyl-tRNA on the peptidyl-tRNA ester (17). A further revision of this model includes catalytic groups, such as histidine, to facilitate the reaction (17). This model is more favorable in light of the lack of evidence to support the first model and from the fact that the peptidyl-tRNA ester can adequately substitute for the acyl intermediate required by the ribosome (17, 18, 19).

The Peptidyl Transferase Reaction

Several years ago, an assay was developed to study the mechanism of peptide bond formation (20, 21, 22). The PTase assay became available when the action of puromycin, acting like an amino acyl-tRNA acceptor and terminating protein synthesis, was understood (23, 24). Puromycin binds to the A site, resembling amino acyl-tRNA, and will covalently attack the peptidyl-tRNA at the P site, through the action of peptidyl transferase, forming a peptidyl-puromycin adduct which can be isolated and quantified (22). The extent of adduct formation provides information about the activity of the PTase Center. This *in vitro* assay is convenient in that only the ribosomal large subunit, a peptidyl-tRNA donor, and puromycin are required for the reaction to occur (22). The need for extraneous factors has been circumvented by the reaction conditions (15, 22). Many of the antibiotics that inhibit protein synthesis do so by binding to the PTase Center, subsequently preventing the peptidyl transferase reaction (6, 25). The binding of these antibiotics is routinely measured by the PTase assay where a decrease in peptidyl transferase activity is detected as a decrease in the puromycin-adduct formed. Classically, this has been an excellent technique for examining the role of chloramphenicol in the inhibition of protein biosynthesis (20, 26).

The Chloramphenicol Binding Site

The antibiotic chloramphenicol (CAP) was first discovered and reported during the late 1940's (27). After its structure was fully elucidated, it became the first antibiotic to have a commercially available synthetic route of production, rather than biosynthetic (28).

The antibiotic property of chloramphenicol was found to be its ability to inhibit protein synthesis within bacteria and further studies provided evidence that the target of action was the ribosome (29, 30, 31).

CAP was later shown to bind with high affinity to a site on the large ribosomal subunit of bacteria and to inhibit the PTase reaction (32, 33). Using similar techniques, the same conclusions were made in regard to the binding of CAP and its mode of action on mitochondrial ribosomes (34).

Properties of Chloramphenicol

Chloramphenicol is a rather small antibiotic with molecular weight of 323. The systematic name for CAP is D-(-)-threo-2-dichloroacetamido-1-para-nitrophenyl-1,3-propanediol. Two interesting functional groups can be noted from inspection of the name, the para-nitro moiety and the dichloroacetate chain. These are unusual features for a biologically synthesized compound and bring about a uniqueness for CAP (28, 35). The molecule CAP can be

divided into three structural parts that are required for its biological activity: 1) para-nitrophenyl ring, 2) dichloroacetate chain, and 3) 2-aminopropanediol chain (29, 35, 36). Each of these positions has been modified, individually or collectively, to produce analogs which have been tested for inhibition of bacterial growth. In this manner, structure-activity relationships for these three positions were ascertained (36). It was proposed that the electronegativity of the nitro moiety and the size and electronegativity of the dichloro moiety played crucial roles in providing for the ability of chloramphenicol to inhibit protein synthesis in bacteria (29, 36). The consensus of the results from various modifications suggest that the CAP binding site can accommodate some minor variation in the nitrophenyl ring and the dichloroacetate chain, but that the 2-aminopropanediol chain must remain stereochemically intact (29, 36, 37). It is of interest that none of the CAP analogs tested attained the inhibitory level of the parent compound, CAP.

Using the structure-activity relationships proposed according to the analyses of the inhibition of bacterial growth by CAP analogs, a derivative of chloramphenicol was synthesized in the expectations that it would be a better antibiotic than CAP (38). When tested though, this analog was shown to be much inferior to CAP (38, 39). As of the present, no derivative of CAP has been found to inhibit protein synthesis in bacteria better than CAP itself. In

the Results and Discussion chapters, I will report some structural modifications of chloramphenicol and the consequences of the changes with respect to the inhibition of the PTase activities of bacterial and mitochondrial ribosomes and I will suggest new structure-activity relationships for CAP and its binding to the ribosome.

Features of the Chloramphenicol Binding Site

Several techniques have been applied to elucidate structural and functional components of the CAP binding site of bacterial ribosomes. Among the more useful techniques have been: 1) the salt-stripping of ribosomal proteins, while following the loss in the ability of CAP to bind to the ribosomes, 2) the reconstitution of the CAP binding activity, by adding individual ribosomal proteins back to protein-deficient ribosomal particles, and 3) the affinity labelling of ribosomal proteins by CAP affinity analogs (40, 41, 42, 43). The ribosomal proteins identified by these procedures have been assigned to be structural and/or functional proteins of the CAP binding site and PTase Center. The bacterial ribosomal proteins L2, L16, and L27 (with molecular weights of about 30,000, 18,000, and 12,000, respectively) are the main proteins selected by these techniques for the PTase Center and CAP binding site from E. coli.

There are features of the CAP binding site that are not readily ascribed to individual proteins. These mainly involve the interactions that allow CAP to bind to the

ribosome. One way of examining for the essential interactions has already been mentioned briefly (see previous section) and has been used primarily by medicinal chemists in search of superior CAP analogs. This procedure entails the selective modification of portions of the CAP molecule and then assaying for the antibiotic activity of the analog in comparison to CAP. The major conclusions of these studies were that the: 1) D-threo conformation is absolutely required, 2) the electronegativity and molar volume are the important features of the dichloroacetate chain, and 3) the electronegativity of the p-nitro moiety is required (29, 35, 36).

NMR spectroscopy has been recently applied to examine the binding of CAP to bacterial ribosomes (44). This technique does not allow one to identify actual components of the CAP binding site, but general features of the binding interactions have been ascertained. From the broadening of peaks in the NMR spectra, it was inferred that the hydroxyls of the aminopropanediol chain of CAP form hydrogen bonds to components of the ribosome in the anchoring of CAP within its binding site on the bacterial ribosome (44).

I will report in the Results and Discussion chapters the important components and features of the chloramphenicol binding site of mitochondrial ribosomes.

Chloramphenicol Resistance

Some bacteria and eukaryotic cells develop the ability to exhibit a resistance to the effects of CAP. The

resistance usually results from the induction of an enzyme that can inactivate CAP or by an alteration of the ribosome so that CAP does not interact in an inhibitory manner with its ribosomal binding site (35, 45, 46). In eukaryotes, CAP resistance generally results from a mutant mitochondrial ribosome and the mutation can be traced to the mitochondrial DNA, where the lesion is localized to the mitochondrial large ribosomal RNA gene (47, 48). Several CAP resistant mitochondrial DNA's have been sequenced and single base mutations have been localized about 300 nucleotides from the 3' end of the large rRNA in resistant cell lines (49, 50, 51). The base substitutions occur in single-stranded regions near the base of a helix in the proposed secondary structure of the rRNA (50, 51). The mechanism of CAP resistance appears to be an alteration in the binding of ribosomal proteins to the affected region of rRNA, thereby decreasing the ability of CAP to bind to the ribosome (51). It is important to note that CAP resistant ribosomes, which show a decrease in the ability to bind CAP, generally exhibit a decrease in their protein synthetic capacity as well (46, 47, 48).

Affinity Labelling of Ribosomes

Traditional Affinity Labelling

Historically, affinity labelling studies were done to determine which amino acid residues may be involved at the active site of the enzyme in question (52, 53, 54, 55). An alkylating or acylating compound was expected to react with

the enhanced nucleophilic moiety at the active site, providing a means of localizing important amino acid residues. The labelling compound could be made active-site-directed by having the same structural groups as the enzyme's substrate (53, 55). Many of the amino acid side groups are potential nucleophiles towards the electrophilic affinity labelling species and the enhanced nucleophilicity of the active site provides a means of increased reaction for the affinity probe (52, 53, 54, 55). As a control for the specific labelling reaction, the enzyme could be incubated with a tight binding substrate prior to the addition of the labelling reagent. In this situation the lack of label on the previously identified amino acid supports the fact that the substrate binding to the active site can effectively block the labelling reaction, and therefore, that the identified amino acid is within the active site (53, 55).

Ribosomal Affinity Labelling

For macromolecular complexes the affinity labelling approach is intended to provide information other than which amino acids are in active sites. Rather, the identification of individual proteins and regions of RNA forming the binding sites is the goal of affinity labelling studies involving ribosomes (56, 57, 58). To this end, various ribosomal substrates have been converted to electrophilic species by de novo synthesis or the addition of an electrophilic moiety to the parent molecule. These

compounds can thus serve as reactive affinity probes towards their respective binding sites on the ribosome. In this way, structural and functional roles can be assigned to proteins that react specifically with particular affinity probes.

Among the first affinity probes used on bacterial ribosomes were two chloramphenicol analogs, iodoamphenicol and bromoamphenicol (59, 60). Iodoamphenicol (IAP) and bromoamphenicol (BAP) were shown to bring about an irreversible inactivation of the bacterial ribosomal protein synthetic capacity that CAP could not (59, 60, 61). Incubation of the ribosome in the presence of [^{14}C]IAP showed covalent incorporation into both subunits upon sucrose gradient analysis (59, 61). Polyacrylamide gel electrophoresis allowed identification of protein L16 from the bacterial ribosome large subunit as the chloramphenicol binding protein (61). Reversible and irreversible inhibition were also shown for BAP (60). Using [^{14}C]BAP, though, L2 and L27 were identified as the primary peptidyl transferase proteins in bacterial ribosomes by another group (60). These early experiments were not carefully controlled, however, and competitive inhibitors were not used to demonstrate blockage of the "specific" labelling reaction. For the IAP experiments a non-specific reagent of similar chemical reactivity, iodoacetamide (IAM), was used in one series of experiments to show a different overall labelling pattern than that obtained from IAP labelling

(61). This control was intended to demonstrate that the enhanced labelling on L16 by [^{14}C]IAP was not simply due to a protein containing a powerful nucleophile.

Substrate analogs, usually substituted tRNA's, have also been used as affinity probes of the Peptidyl Transferase Center (56, 57, 58). Although more difficult to prepare and purify, these tRNA affinity probes have a much higher affinity for the Peptidyl Transferase Center and thereby, potentially, a much more specific reaction. The tRNA affinity probes have labelled many proteins including L2, L27, and L16, (56, 57, 58). These proteins are probably intimately associated with the Peptidyl Transferase Center and most likely are very close to the actual CAP binding site of bacterial ribosomes, as revealed by their labelling by CAP affinity analogs.

The Basic Approach to Affinity Labelling the PTase Center and the CAP Binding Site

In this section, I have compiled the general criteria that should be used in the affinity labelling study. Previous studies using affinity labelling to identify the proteins involved in the CAP binding site have been somewhat ambiguous because of the lack of complete experimental evidence. I have sought to overcome the weaknesses of previous studies by following the guidelines listed below.

Reversible Binding

To be useful in an affinity labelling study, the affinity probe must be able to interact with its binding

site in a manner similar to its parent compound. That is, any derivatization must not abolish the active site direction of the parent molecule. For the peptidyl transferase activity, reversible inhibition can be measured for the antibiotic and its affinity probe derivative by the peptidyl transferase assay. In this way, a functional interaction with the Peptidyl Transferase Center can be ascertained for the affinity probe, providing a rationale for continued testing of its utility as an affinity probe. For electrophilic affinity probes, the time of exposure with the ribosomes is minimized to limit the amount of covalent adduct formation and, therefore, irreversible inactivation that would be measured as reversible binding in the peptidyl transferase assay. For similar reasons, photo-affinity probes would be assayed in the dark. Reversible binding to the Peptidyl Transferase Center is one of the most useful criteria for a functional interaction with the ribosome.

Irreversible Inactivation

It would be expected that if a binding site were occluded, then a function associated with the site would diminish. This could occur with saturating amounts of a reversible inhibitor under which conditions the site is effectively always occupied or by the formation of a covalent linkage within the site so that the site is permanently occupied by the covalently-attached probe. The electrophilic affinity probe can occlude its binding site if a properly oriented nucleophilic group in the binding site

can react with the probe, whereas a photo-affinity probe requires only excitation by light to bring about covalent attachment within the site (55). Total inactivation of peptidyl transferase could be expected if the affinity probe and its binding site meet the above criteria. In actuality, for a correctly oriented nucleophile to be present on the ribosome is an unlikely event, because of lack of the "classical active sites" containing nucleophilic amino acids. And since photo-incorporation rarely proceeds to saturation total inactivation of the PTase activity seems unlikely (17, 58).

Labelling Parallels Irreversible Inactivation

When the conditions for interaction with the binding site by the affinity probe have been fulfilled and the probe is available with a radioisotope incorporated into its molecular structure, a series of experiments can be undertaken to study the covalent attachment of the affinity probe to ribosomal components. The "hot" affinity probe is incubated with the ribosomes for various periods of time, using the concentrations previously determined to produce a response in the ribosomal peptidyl transferase activity. The extracted ribosomal proteins, separated by unidimensional and bidimensional gel electrophoresis and subjected to fluorography, are then examined for the extent of covalently-attached label. Ideally, specific labelling (active site directed labelling) is expected to occur with increasing time or probe concentration in a manner that

parallels the time-concentration dependence of the irreversible inactivation of the peptidyl transferase activity. Under these conditions the extent of label appearing in a specific ribosomal protein should reflect the extent of irreversible inactivation of the ribosome (55). That is, the loss of activity should correlate with the amount of affinity probe on the protein being labelled.

Labelling Saturates Mole Per Mole

The ribosomal peptidyl transferase activity involves one region of the large subribosomal particle. The labelling via an affinity probe for a protein in a functional binding site is expected to saturate at a stoichiometric relationship of one affinity probe molecule on a protein, if only one reactive nucleophile is present on the protein. Ideally, the labelling kinetics are expected to correlate with the kinetics of irreversible inactivation of the Peptidyl Transferase Center (55). Saturation of labelling in this manner implies a unique point of attachment in the site for the affinity probe and a specific relationship for the probe and the site. For proteins not associated with the binding site, a non-saturating labelling pattern will be observed, which resembles a non-affinity directed labelling pattern as observed with a general labelling reagent (55).

Competitive Inhibition of the Affinity Labelling Reaction

Definitive assignment of a ribosomal protein to the Peptidyl Transferase Center occurs when the labelling of the protein by an affinity probe can be blocked by a competitive binder to the same site. The best examples would involve utilizing the parent molecule to block a derivatized compound synthesized from the parent molecule, such as chloramphenicol, to competitively block the CAP affinity analogs.

Once interaction with the ribosome has been shown for an affinity probe and labelling on a protein via the probe saturates in a manner that can be blocked by a competitive inhibitor, then assured assignment of the protein to the ribosomal Peptidyl Transferase Center can be made. Having met these criteria successfully for chloramphenicol affinity analogs, one will be able to identify proteins at the CAP binding site.

Problems Associated with Affinity Labelling

Affinity probes can be classified into two categories, photo-affinity probes and electophilic affinity probes, each with special problems. Photo-activatable affinity labelling reagents in theory are the best active site labellers to be found, but in practice have not worked that well with the ribosome (58). The affinity labelling reaction is time, concentration and light fluence dependent for the photo-affinity probe. It may also be nucleophile dependent,

if an electrophilic species is the light-generated product. The chemistry of photo-addition is largely unknown and a variety of reaction and rearrangement products may be produced. Reactions with water and buffer may also occur as predominant side reactions, competing with active site labelling. The rapid exchange of CAP or its photo-affinity analogs with the CAP binding site may be fast enough to allow a "photo-activated" intermediate in solution to diffuse into the CAP binding site and then covalently attach to protein, but the "photo-activated" probe may diffuse away from the site before covalency occurs. The reaction of photo-activated probes with the Peptidyl Transferase Center, though, should be enhanced by the active site direction of the photo-affinity analog, but the probe may act as a competitive blocker of itself if non-activated probe is bound at the site.

Electrophilic affinity labelling reagents have another problem, in that all nucleophiles on ribosomal proteins will react with the affinity probe according to their accessibility on the ribosome. Also, water or buffers may react with the affinity probe effectively decomposing it, before sufficient labelling can occur. Furthermore, thiol agents such as 2-mercaptoethanol (2ME) certainly will react exceptionally well toward most electrophilic reagents, so the ribosomes must be free of 2ME prior to affinity probe incubation. Very powerful nucleophiles on ribosomal proteins at locations other than the Peptidyl Transferase

Center and the CAP binding site may also react with the affinity probe, giving a false positive affinity labelling reaction. However, as a control for this reaction, the competitive blockage by the parent compound should not affect the labelling on these proteins, making the issue less confusing. An equally important consideration is that a correctly oriented nucleophile is required in the binding site of chloramphenicol for each particular electrophilic affinity probe used. If this in fact were the case, then complete, irreversible inactivation of the peptidyl transferase activity could be achieved at the saturation of labelling in the CAP binding site. Such correctly oriented nucleophiles would be fortuitous and the amount of irreversible inhibition will depend upon the proximity of the labelled amino acid side group to the CAP binding site.

Chloramphenicol exchanges rapidly at its binding site on E. coli ribosomes (44). Since mammalian mitochondrial ribosomes and bacterial ribosomes have a similar dissociation constant (K_d) for CAP, it is expected that CAP will also exchange rapidly at the mitochondrial ribosomal binding site (34). The affinity probe should be exposed to nucleophilic amino acid side groups as it moves in or out of the CAP binding site so that "affinity" directed labelling is enhanced on proteins with available nucleophiles in or near the CAP binding site. The disadvantage of not having an extremely low K_d is that a higher concentration of affinity probe is required to allow

significant labelling of the CAP binding site, and this may lead to more non-specific labelling of nucleophiles on the ribosome surface. Also a concentration of CAP at 4 mM or higher is required to completely obscure the CAP binding site as a competitor of the affinity labelling probe (44).

Another problem is that the modification of chloramphenicol to produce an affinity probe may also bring about the loss of binding to the CAP site. This is easily discovered in the PTase assay testing procedure and provides valuable information about the positions of the CAP molecule pertinent for binding and inhibition of protein synthesis.

Summary

Mammalian mitochondrial ribosomes are similar to bacterial ribosomes in their general features, but differ markedly in their specific properties. One common feature is the binding of chloramphenicol and thereby the inhibition of the peptidyl transferase activities of the ribosomes. The CAP binding site can be studied by various techniques to identify structural and functional components of the site. The most useful techniques involve modification of chloramphenicol and examining the CAP analogs as inhibitors of the PTase activity or as affinity probes for the CAP binding site. These procedures have allowed the identification of proteins at the bacterial CAP binding site. In addition, features of the CAP molecule that are required for binding, and the complementary ribosomal

features required for the binding of CAP have been identified.

In the present study, I will report the ribosomal proteins from mammalian mitochondria involved with the CAP binding site and propose a topological model that maps the position of these proteins. From the analyses of the binding of CAP and the CAP analogs to the ribosomes, I have developed a model describing the features of chloramphenicol that are required for CAP to bind to the chloramphenicol binding site.

CHAPTER II
MATERIALS AND METHODS

A list of the buffers and their compositions appears in Appendix I. Appendix II contains the standards for quantifying ribosomes by UV spectroscopy and the molecular weights of the chloramphenicol analogs.

Ribosome Preparation

Mitochondrial Ribosomes

The mitochondrial ribosomes were prepared essentially as has been previously reported (2, 3, 8, 13, 34). All preparations began with livers of freshly killed cows transported to the laboratory in ice. Four to eight kg of fresh liver were ground in a meat grinder and diluted with four volumes of Buffer A or B. Subsequently, the mixture was passed through a coarse-mesh plastic screen and homogenized by a high-frequency cavatation device (a Tekmar Super Dispax, Model SD-45K).

Unbroken cells and nuclei were removed from the mitochondria by pumping the homogenate at 880 ml/min through a Vernitron CFR-2 continuous flow rotor spinning at 10,000 rpm in a Vernitron LCA-2 centrifuge or by centrifuging the homogenate in individual buckets of a JA-10 rotor in a

Beckman J-21 centrifuge at 3,000 rpm for 10 minutes. The mitochondria were harvested from the combined supernatants by continuous-flow centrifugation in a Beckman JCF-Z rotor at 18,000 rpm with a flow rate of 440 ml/min or by pelleting the mitochondria in the buckets of a Beckman JA-10 rotor at 10,000 rpm for 10 minutes. The mitochondria were washed three times by resuspension into Buffer A and centrifugation at 10,000 rpm for ten minutes in a JA-10 rotor.

To isolate the ribosomes, the mitochondria were resuspended to a concentration of 20 mg protein/ml in Buffer C and lysed by the addition of Triton-X100 to a final concentration of 2%. The resulting lysate was clarified by centrifugation in a Beckman JA-10 rotor at 10,000 rpm for 45 minutes. Fibrous DEAE (DE52, Whatman) was added to the lysate, at the amount of 1 g DEAE per 1 g of mitochondria. After stirring for 30 minutes, the fluid was filtered from the DEAE. The ribosomes are eluted from the DEAE with Buffer D. The ribosomal solution was concentrated by ultrafiltration in a DC-10 Amicon Hollow Fiber Filter ultrafiltration device to a volume of one liter. The ribosomes are pelleted by centrifugation for 20 hours at 35,000 rpm in a Beckman type 35 rotor through a 45% sucrose cushion of Buffer E. The ribosomal pellets were resuspended into 10 ml Buffer E containing puromycin and incubated for five minutes at 37°C to remove any nascent polypeptide chains from the ribosomes. The ribosomal solution was clarified by centrifugation in a Beckman type 65 rotor for ten minutes

at 20,000 rpm. The supernatant was loaded onto a zonal gradient (Beckman Ti 14 rotor) of 10% to 30% (linear) sucrose in Buffer F for the 55S monosome preparation or of Buffer G to dissociate the 55S into 39S and 28S subunits. The ribosomes were centrifuged for 12 hours at 30,000 rpm in the former case and for 17 hours at 30,000 rpm for the latter case. The gradient was fractionated into 20 ml fractions collected by an LKB 2112 fraction collector while being continuously monitored by a Uvicord UVA ultraviolet monitor. The fractions containing ribosomes were pooled and centrifuged for 16 hours at 50,000 rpm in a Beckman Ti 60 rotor. The pellets were resuspended into Buffer H and aliquoted for storage at -70°C.

E. coli Ribosomes

E. coli ribosomes were prepared by Mary Conde as reported (34). E. coli K-12, strain Hfr DIO RNase- or strain 1200F- end A 1100 rns A Sn- were grown to early exponential phase in nutrient broth at 37° in a rotory shaking incubator. The cells were harvested by low speed centrifugation (JA-10 Beckman rotor, 6,000 rpm for ten minutes). The cells were resuspended in 10 mls of cold Buffer I and sonicated three times of 20 seconds each. The cellular debris was removed by centrifugation (Beckman type 65 rotor, 15,000 rpm for 10 minutes) after which the supernatant was centrifuged in a Beckman type 65 rotor for two hours at 50,000 rpm. The 50S ribosomal large subunits were prepared by placing 20 to 30 A_{260} of 70S ribosomes

into 2 ml of Buffer J, loading the solution on the top of a 10% to 30% linear sucrose gradient of Buffer J in a Beckman SW 27 rotor and centrifuging at 27,000 rpm for nine hours. The gradients were fractionated under continuous monitoring by a Gilford 2400 spectrophotometer. The fractions containing subunits were pooled and centrifuged in a Beckman type 65 rotor for two hours at 50,000 rpm. The pellets were resuspended into Buffer K and stored frozen at -70°C.

Thin Layer Chromatography (TLC)

The synthetic reactions of CAP analog production required careful monitoring. I used silica gel TLC plates (Merck 60 F 254) with $CHCl_3$:CH_3OH (95:5, 9:1 or 3:1) and ethyl acetate as the eluants for the monitoring steps and to help gauge the purity of the products. Each analog was developed on several chromatograms in the different solvent systems until I was convinced that the product was clean from reactants or unwanted products. In most cases a 10 to 100 fold excess of the analog was spotted on the plates to reveal less than 10% contamination. In conjunction with other analyses such as melting points, UV spectroscopy, IR spectroscopy, NMR spectroscopy, and Mass spectroscopy all of the analogs appeared to be greater than 99% pure, by having less than 1% contaminants.

Synthesis of the CAP Analogs

Iodoamphenicol (IAP) (D-threo-2-iodoacetamido-1-p-nitrophenyl-1,3-propanediol)

Iodoamphenicol was synthesized essentially according to published procedures (59,62). Iodoacetic acid (Aldrich) was recrystallized and stored in the dark under desiccant at -20°C. The iodoacetic acid (1.0 g) was dissolved into 25 ml dry dioxane and N-hydroxysuccinimide (Sigma) (0.7 g) was added. Dicyclohexylcarbodiimide (Aldrich) (1.3 g) in 10 ml dry dioxane was added to the mixture. The reaction was allowed to proceed at room temperature for one hour in the dark after which the dicyclohexylurea was removed by centrifugation. The supernatant was dried by rotary evaporation and recrystallized from isopropanol. Chloramphenicol base (Sigma) (0.5 g) was dissolved into 20 ml cold ethyl acetate (0°C). Iodoacetylsuccimide ester (0.7 g) was added slowly over several minutes and the mixture was stirred at 0°C for two hours. An additional one hour at room temperature incubation followed after which 50 ml ethyl acetate and 50 ml 0.1 N H_2SO_4 were added to provide a two-phase extraction system. The organic phase was obtained and worked up to provide iodoamphenicol. TLC analysis showed a single resolvable spot which migrated to a distinct position slightly slower than where CAP does in the solvent systems employed. Alkaline reaction conditions and aluminum oxide as the chromatography medium were avoided since these tend to cause the decomposition of IAP into a

morpholino adduct by the internal hydroxyl attack at the iodo-containing carbon.

[^{3}H]Iodoamphenical was synthesized in a micro-scale reaction, scaled down from the one listed above. [^{3}H]Iodoacetic acid (100 mCi/mmole, Amersham) was the starting material. Instead of a two-phase separation, the crude [^{3}H]IAP was applied to a silica gel (Merck) column (2.5 cm x 10 cm) and eluted with $CHCl_3$:CH_3OH (9:1). One-half ml fractions were collected and the [^{3}H]IAP containing fractions were pooled and concentrated. The [^{3}H]IAP residue was dissolved in 95% ETOH and stored in the dark at -20°C. TLC analysis was periodically performed prior to affinity labelling reactions to check for decomposition of the [^{3}H]IAP.

p-Amino-chloramphenicol (ACAP)
(D-threo-1-p-aminophenyl-2-dichloroacetamido-1,3-propanediol)

ACAP was synthesized from CAP (Sigma) by sodium dithionite reduction (63). CAP (5 g) was placed in 100 ml 0.1 M $NaHCO_3$, pH 7.0, and sodium dithionite was added slowly. The reduced CAP was extracted by ethyl acetate (3 x 100 ml) or was acidified and precipitated as the ACAP HCL. The ACAP HCL was sometimes neutralized and extracted into ethylacetate for recrystallization. ACAP was somewhat yellow in color and remained near the origin on silica gel TLC analyses. ACAP produced in this manner was used in the PAP and FAP syntheses.

p-Azido-chloramphenicol (PAP)
(D-threo-1-p-azidophenyl-2-dichloroacetamido-1,3-propanediol)

PAP was synthesized from chloramphenicol (Sigma) as the starting material. CAP was reduced by hydrogen gas over activated pallidium-charcoal or by sodium dithionite reduction (63, 64, 65). The reduced CAP was diazotized by $NaNO_2$ (0.25 g) in 50 ml 20% H_2SO_4 at 0°C for 30 minutes after which sodium azide (0.5 g) was added followed by a 30 minute incubation at 0°C. A very pure PAP was recovered from the aqueous phase by three ethyl acetate extractions (50 ml each). The extracts were dried to a yellow oil which was recrystallized from isopropanol to give a very clean product. All reactions were carried out in th dark to prevent photolysis of the PAP. I performed a stability test by allowing PAP in methanol to remain in a sealed glass vesicle on the countertop. It took more than three days of normal room light exposure to provide a significant decomposition of PAP. The UV spectrum of PAP was very useful in determining its purity since it is very distinct from CAP, and the photolytic reaction could be followed spectrophotometrically (see Appendix III), further distinguishing PAP from CAP.

p-Azidobenzoamido-chloramphenicol (FAP)
(D-threo-1-p-azidobenzoyl-p-amidophenyl-2-dichloroacetamido-1,3-propanediol)

For the synthesis of FAP and chloramphenicol-p-azidobenzoate (PAF), the activated ester of p-azidobenzoic acid was required. All reactions were performed in the dark to prevent photo-decomposition of the materials.

p-Azidobenzoic acid was synthesized from p-aminobenzoic acid (Calbiochem) similar to the manner that PAP was synthesized. p-Aminobenzoic acid (0.5 mole) was dissolved in 300 mls of cold water with 100 ml H_2SO_4. $NaNO_2$ (0.6 mole) in 150 ml cold water was added portion-wise over 30 minutes with continuous stirring. After an additional one hour of stirring at 0°C, sodium azide (1.0 mole) in 100 ml cold water was added portion-wise over 30 minutes and then allowed to react an additional one-half hour. The tan, mud-like product was filtered and recrystallized from ethanol. TLC analysis gave one resolvable spot and the UV spectrum was consistent with p-azidobenzoic acid (66).

The active ester of p-azidobenzoic acid was synthesized in a like manner to the synthesis of the active ester of iodoacetic acid. p-Azidobenzoic acid (24.5 g) and N-hydroxysuccinimide were dissolved into 250 ml of dry dioxane and chilled on ice to 0°C. Dicyclohexyl-carbodiimide (31 g) in 50 mls of dry dioxane at 0°C was added to the mixture and the solution was allowed to warm to room temperature and incubate overnight. The

dicyclohexylurea was removed by filtration and the orange filtrate was evaporated to cream colored crystals which were recrystallized from dioxane.

FAP was synthesized from ACAP (10.0 mmole) and p-azidobenzoylsuccinimide ester (10.0 mmole) in 50 ml of ethyl acetate. The reaction was performed at 0°C for two hours and subsequently overnight at room temperature. The product had precipitated as an orange clay-like material from the ethyl acetate. This was washed several times with water and then dissolved in DMSO. TLC and spectral analyses showed that the FAP was free from contaminating materials. (Photolytic experiments with FAP are given in Appendix III.)

Chloramphenicol-p-azidobenzoate (PAF)
(D-threo-2-p-azidobenzoamido-1-p-nitrophenyl-1,3-propanediol)

PAF was synthesized from CAP base (Sigma) (10.0 mmole) and from p-azidobenzoylsuccinimide ester (10.0 mmole) according to the scheme given for FAP. The reactants were dissolved in cold ethyl acetate (50 mls) and incubated for two hours at 0°C and then overnight at room temperature. Likewise an orange clay-like precipitate occurred which was washed with water and then dissolved in DMSO. TLC and spectral analysis showed PAF to be free from contaminants.

p-Hydroxylamino-chloramphenicol (HACAP) (D-threo-2-dichloracetamido-1-p-hydroxylaminophenyl-1,3-propanediol)

HACAP was synthesized from CAP essentially following the published procedure (67). CAP (10 g) and NH_4Cl (3 g) were vigorously stirred in 120 ml of 25% ethanol. Zinc dust (7.8 g) was added slowly over 10 minutes after which the solution was allowed to stir an additional 15 minutes. Thirty ml of water was added and the suspension was filtered. The filtrate was saturated with NaCl and extracted three times with 150 ml ethyl acetate. The extracts were dried and the residue was dissolved in 95% ethanol. UV and NMR spectral analyses exhibited results that were identical to the published spectra of HACAP (67). HACAP was stored at -20°C until used.

p-Nitroso-chloramphenicol (NOCAP) (D-threo-2-dichloroacetamido-1-p-nitrosophenyl-1,3-propanediol)

NOCAP was also synthesized essentially following the published procedure (67). CAP (10 g) was reduced to HACAP as described above except the procedure was ended prior to ethyl acetate extraction. The aqueous phase containing the HACAP was chilled on ice to 0°C and poured slowly into 100 ml of $FeCl_3 \cdot 6H_2O$ (20 g) at 0°C. The solution immediately turned green. An additional 30 minutes of stirring at 0°C was allowed. The solution was extracted three times with 75 ml of diethyl ether. The ethereal phase was evaporated to a blue-green residue. The residue was chromatographed on a silica gel (Merck) column (2.5 cm x 30

cm) with $CH_3Cl:CH_3OH$ (95:5) as the eluent. A blue band trailed by a yellow band were resolved and the blue band was collected and recrystallized from diethyl ether to give pale blue crystals. UV spectroscopy gave a spectrum identical to authentic NOCAP (67). [^{14}C]NOCAP was synthesized from [^{14}C]chloramphenicol (14.9 mCi/mmole) (Amersham) in a reaction scaled down from the scheme given above. The [^{14}C]NOCAP migrated coincident with the unlabelled material with no radioactivity elsewhere on the TLC plate (silica gel, $CH_3Cl:CH_3OH$, 95:5).

The Peptidyl Transferase Assay

The PTase assay employed has been published in part previously (34). 0.5 to 1.0 A_{260} of ribosomes were required for each data point to be collected in the assay. Each concentration of antibiotic was assayed in duplicates or triplicates which were averaged for the final value. Many of the antibiotics were assayed on several different occasions with no change in the expected inhibition value. The ribosomes were placed in 90 µl of Buffer K to which 50 µl of 95% ETOH or 50 µl of antibiotic solution in 95% ETOH was added. The reaction was initiated by the addition of 10 µl of N-acetyl-[^{3}H]leucyl-tRNA (10,000 cpm) to the ribosomal mixture (total volume 150 µl). (The N-acetyl-[^{3}H]leucyl-tRNA was received as a gift from Nancy Denslow.) The reaction was allowed to proceed for ten minutes at room temperature and terminated by the addition of 10 µl of 10 N KOH. A further incubation for three

minutes at 40°C followed. The solutions were neutralized by the addition of 1 ml of 1 M sodium phosphate pH 7.0. Each sample was extracted with 1.5 ml ethyl acetate and the organic phase was separated from the aqueous phase by low speed centrifugation. One ml of the upper phase, which contained the N-acetyl-[^{3}H]leucyl-puromycin adduct, was collected and added to 10 ml ACS (Amersham) liquid scintillant and then the samples were quantified by liquid scintillation counting. Sample reactions without ribosomes were used to register the background and reactions with ribosomes but without antibiotic present were used to give the untreated control values. The results are presented as a percentage of the untreated control after background subtraction. The antibiotic solutions were made fresh prior to each assay and assays were conducted in subdued ambient lighting to prevent any photolytic decomposition of the antibiotics during the assay.

Apparent Dissociation Constant (K'_d) Calculations

As a measure of how well the CAP analogs could interact with the ribosomes, an apparent dissociation constant (K'_d) was calculated from the PTase inhibition data using the method of Denslow and O'Brien (34). The method transformed the data into a Scatchard format so that the K'_d could be evaluated from the slope (68). The formula of transformation is: $i/[I] = -(i/K'_d) + (n/K'_d)$, where i is the fraction of inhibition defined as 1-(percent of control/100), [I] = antibiotic concentration, n = number

of binding sites, and K'_d = apparent dissociation constant. From the plot of i/[I] versus i, the negative of the reciprocal slope (-1/slope) is the K'_d and n, the abscissa intercept is the number of binding sites. The slope was evaluated by linear regression using a Texas instrument SR-51-II hand-held calculator.

Structure-Activity Relationship of CAP Analogs

Previously, CAP analogs have been examined for a structure to activity relationship, primarily by medicinal chemists (29, 35, 36, 37, 38). In some instances, the Hansch equation has been used to correlate the structure to the activity and in others a measure of a physical feature of the structure has been plotted versus the measured activity to examine for correlations (36, 38, 69, 70). I chose the latter route and found literature values for the properties of the para substituents. The Hammett sigma values were used as a measure of the electronegativity and were 0.78 for the nitro, 0.15 for the azido, 0.12 for the nitroso, -0.34 for the hydroxylamino, and -0.66 for the amino groups (71). The shift in the infrared absorbance frequency upon hydrogen bond formation was used as a measure of the hydrogen bond strength; these values were 346 cm^{-1} for the nitro, 240 cm^{-1} for the nitroso, 175 cm^{-1} for the amino, and 40 cm^{-1} for the azido groups (72, 73). The infrared frequency shifts (i.e. hydrogen bond strengths) had been measured in a manner which would be similar to the situation involved when CAP is in its binding site. The activities

of the ribosomes were computed from the K'_d values obtained from the PTase assays using the formula: log Act = log [(K'_d of CAP/K'_d of CAP analog) x 100], where K'_d of CAP is 38 μM and 9.8 μM for the mitochondrial and bacterial ribosomes, respectively, and K'_d of CAP analog represents the calculated K'_d of the CAP analog tested. This equation is a transformation of an equation used previously to calculate CAP analog activities (38).

Labelling Reactions

The mitochondrial ribosomes were retrieved from the -70°C freezer and suspended in Buffer L, which lacks 2-mercaptoethanol (2ME). The ribosomes were centrifuged at 30,000 rpm for 16 hours in a Beckman type 65 rotor over a 20% sucrose cushion of Buffer L to remove traces of 2ME prior to the labelling reactions. The supernatant was discarded and the ribosomal pellets were resuspended into Buffer L. A small aliquot was removed for an A_{260} measurement and the ribosome concentration was adjusted to give a final concentration of 1 A_{260}/10 μl after the addition of the labelling reagent. The labelling reagent [^{3}H]IAP (100 mCi/mmole), [^{14}C]NOCAP (14.9 mCi/mmole) or [^{14}C]iodoacetamide (14.7 mCi/mmole), was added to the ribosome solution in 95% ETOH to give a final ETOH concentration of 10%. In the reactions including CAP as the competitive blocker, one-half of the ethanol added contained CAP and the other half contained the labelling reagent.

N-Acetyl-AA-tRNA and AA-tRNA were a gift from Nancy Denslow for use in the [^{3}H]IAP affinity labelling studies and were added to the labelling reaction solution in water. The AA-tRNA may contain some uncharged tRNA and the N-acetyl-AA-tRNA was greater than 95% acetylated, so that AA-tRNA may not be fully confined to the A site, but the N-acetyl-AA-tRNA would be expected to bind mainly to the P site. Regardless, they will still be binding to a restricted region of the ribosomal surface. The labelling reactions were terminated by the addition of a large excess of 100 mM 2ME in Buffer L (7 ml) or in 95% ETOH (1 ml). Reactions terminated with 100 mM 2ME in Buffer L were centrifuged at 30,000 rpm for 16 hours in a Beckman type 65 rotor to concentrate the ribosomes. The reactions terminated by the addition of 100 mM 2ME in 95% ETOH were chilled, and the ribosome precipitate was collected by centrifugation for 20 minutes in a Beckman Microfuge. The ribosomes were resuspended into an appropriate buffer for the extraction of the ribosomal proteins for polyacrylamide gel electrophoresis.

Sucrose Density Gradient Separation of Labelled Ribosomal Subunits

Subsequent to the [^{3}H]IAP labelling of the 55S ribosomal monosome, a separation and isolation of the individual subunits were required. The labelled ribosomal pellets were resuspended in 2.0 ml Buffer G to bring about monosome dissociation into subunits. The resuspended

ribosomes were loaded on a linear 10% to 30% sucrose gradient of Buffer G in a Beckman SW 27 rotor and centrifuged at 23,000 rpm for 18 hours. The gradient was fractioned under continuous UV monitoring at 260 nm by a Gilford 2400 UV spectrophotometer. The fractions containing subunits were pooled and centrifuged at 30,000 rpm (Beckman type 65 rotor) for 22 hours to concentrate the ribosomal subunits. The ribosomal pellets were resuspended in 50 µl of Buffer G and an aliquot was removed for an absorbance measurement. Equal amounts of ribosomal subunits were then prepared for electrophoresis.

Polyacrylamide Gel Electrophoresis (PAGE)

One-Dimensional SDS PAGE

Unidimensional PAGE was conducted using a modified Laemmli system (74, 75). The modifications consisted of doubling the separator gel buffer (Buffer M) concentration and the stacking gel buffer (Buffer N) concentration, which helped to prevent streaking artifacts of the migrating proteins. The polyacrylamide concentration in the separator gel was 15.6% T 3.3% C and the stacking gel was 10% T 2.6% C, to provide resolution of proteins from approximately 8000 MW to 70,000 MW. The slab gels measured 130 mm x 240 mm x 1.5 mm and were electrophoresed on a two-slab laboratory made unit. Ammonium persulfate was added at 0.03% and 0.1% to initiate polymerization of the separator and stacking gels, respectively. The separator gel was overlayed with isobutanol after the polymerization was

initiated to provide an even surface for the stacking gel. Residual isobutanol was washed away prior to the addition of the stacking gel. The separator gel was allowed one full hour to polymerize before the stacking gel was added, which was also allowed to polymerize with a ten-well comb in place for one hour.

The ribosomal pellets were suspended in Buffer O and heated at 60°C for at least 30 minutes prior to loading onto the gels. Tank buffer (Buffer P) was used to flood the wells of the stacking gel and the samples consisting of 20 µl to 50 µl each were underloaded through the tank buffer via micropipets and a hand-held micro-pipetting device. The PAGE was conducted at an initial constant current of 15 mA per gel (30 mA for two gels) and was increased to 20 mA per gel (40 mA total constant current) after the Bromophenol Blue tracking dye had fully entered the gel. The voltage routinely began at about 70 volts which plateaued at about 220 volts near the termination time. The gels were electrophoresed for 16 to 24 hours or for about two hours after the tracking dye had migrated off the bottom of the gels. The gels were removed after termination and placed in a fix bath of 25% isopropanol and 10% acetic acid overnight to remove the SDS. Subsequently, the gels were stained with 0.25% Coomassie Brilliant Blue R-250 in 50% ethanol and 7.5% acetic acid for four to six hours. The gels were destained by repeated changes of a destain solution of 5% ethanol and

10% acetic acid. After sufficient destaining, the gels were prepared for fluorography (see below). The outside lanes were used to resolve molecular weight standards, which had been obtained from Sigma and Calbiochem. They consisted of 1) bovine serum albumin, 68,000 MW; 2) ovalbumin, 44,500 MW; 3) human gammaglobulin, 50,000 MW and 23,500 MW; 4) carbonic anhydrase, 30,000 MW; 5) myoglobin, 17,200 MW; 6) lysozyme, 14,400 MW; and 7) bovine cytochrome C, 12,500 MW, which were stored in Buffer O. Molecular weights of ribosomal proteins were calculated from a plot of the migration position of these standards versus their molecular weights (76).

Two-Dimensional PAGE

Bidimensional PAGE was conducted using the Leister and Dawid system as has been previously modified in our laboratory (13, 77, 78, 79). The ribosomal pellets obtained from the labelling reaction were resuspended in 50 µl of Buffer L and 65 mg of high purity urea (Beckman), 15.3 mg of LiCl, 2 µl of 1 N HCl, and 1 µl of 10% butylatedhydroxy-toluene in ethanol were added after which the volume was adjusted to 120 µl. The final extraction solution consisted of 9 M urea and 3 M LiCl with a pH of about three. The extraction mixtures were allowed to stir overnight (about 16 hours) in small conical vials at 4°C. The rRNA was removed from the extraction solution by centrifugation at 50,000 rpm in a Beckman type 65 rotor for one hour. The rRNA precipitate was re-extracted in

80 µl of 8 M urea, 3 M LiCl, pH 3.5 for about ten hours to dislodge any ribosomal proteins that had become entrapped in the rRNA precipitate. The combined supernatants were dialyzed overnight against sample buffer (Buffer Q).

The first dimension gel was designed to resolve basic proteins and was formed in a tube 2 mm in diameter and 120 mm long. The gel consisted of a separator gel (4.6% T 3.2% C), stacking gel (4.0% T 3.5% C), and a sample gel (4.7% T 3.5% C) of Buffer R, Buffer S, and Buffer T, respectively. The separator gel was polymerized by the addition of 0.1% total ammonium persulfate and was pre-electrophoresed in Buffer Q at 0.2 mA per gel overnight, before the addition of the stacking and sample gels, which contained riboflavin (0.001%) in addition to ammonium persulfate (0.02%) so that the acrylamide could be induced to photo-polymerize. Pyronin Y (0.02%) was used as the tracking dye and the gels were electrophoresed under constant current towards the cathode at 0.2 mA per gel until the dye was one centimeter into the separator gel whereupon the current was increased to 0.5 mA per gel. The tank buffer was buffer U. The run was terminated when the tracking dye reached the bottom of the tubes.

The second dimension gels were slab gels (180 mm x 200 mm x 1.5 mm) consisting of a separator (Buffer V) and stacking (Buffer W) gels of 10% T 3.5% C and 4% T 3.5% C acrylamide concentrations, respectively. The first dimension tube gel

was extruded from the glass tube by water pressure, placed on the top of the slab gel, and allowed to polymerize into the stacking gel. Molecular weight marker proteins were placed in wells on both sides of the tube gel along with an agarose plug of Bromophenol Blue tracking dye. The second dimension gels were electrophoresed at 40 to 45 mA, in Buffer X (tank buffer), until the dye reached the bottom of the gels, generally after about 24 hours. Upon termination, the gels were fixed and stained as described above.

Fluorography

The stained polyacrylamide gels were originally prepared for fluorography by the method of Bonner and Laskey (80). The gels were dehydrated by two washes of 1.2 liters DMSO for 30 minutes each, then 230 ml of a 20% solution of 2,5-diphenyloxazole (PPO) in DMSO was added for three hours to impregnate the gels with fluor. Finally, the fluor was precipitated in the gels by a one hour water wash. The fluor-impregnated gels were dried under heat and vacuum on filter paper (Whatman 3MM) by a Biorad gel dryer for 1.5 hours. More recently the gels were impregnated with sodium salicylate as the fluor (81). The gels were washed in water for about 30 minutes to remove the destain solution. Next the gels were soaked in ten volumes of 1 M sodium salicylate pH 6-7 for 30 minutes after which they were dried as above.

The dried, fluor-impregnated gels were placed directly against Kodak XRP-5 medical x-ray film which had been pre-

flashed with a photographic strobe as described by Laskey and Mills (82). The cassettes containing the gels and film were stored at -70°C for exposure. After an appropriate time interval the film was removed and developed in an automated x-ray developer.

Densitometry

The fluorograms obtained from the labelled ribosomal protein gels were scanned by a light transmission Densitometer (EC 910), while the tracings were recorded on a Heathkit strip-chart recorder.

Cutting and Digestion of Protein Bands or Spots from Polyacrylamide Gels

The stained protein bands or spots were cut from the dried gels using a small pair of scissors. The paper backing was removed and the dry plugs were placed in screw-type scintillation vials. Hydrogen peroxide (200 µl) was added to each vial to hydrate and partially digest the gel plugs. After one or two days of hydration at room temperature the vials were frozen and 500 µl of NCS (Amersham) was added to solubilize the labelled protein from the residual of the gel plugs. After one or two more days of soaking at room temperature, the gel plugs were usually totally digested and free of color. Once again the vials were frozen and then 10 ml ACS (Amersham) liquid scintillation cocktail was added to each vial. The samples were allowed to become quiescent before liquid scintillation counting by storage in the dark for two or three days.

Radioactive standards were added to vials containing gel plus of the molecular weight marker protein to examine for the loss of radiolabel during the digestion procedure, which was generally less than 5%.

Liquid Scintillation Counting (LSC)

Liquid scintillation counting was conducted in a Beckman LS8000 liquid scintillation counter using the preselected programs. The counting efficiency was generally about 20% for tritium and about 75% for carbon-14.

CHAPTER III

RESULTS

It has been known for some time that chloramphenicol can inhibit protein synthesis within mitochondria, but very little has been reported about the interaction of CAP with the ribosomes from mammalian mitochondria (83, 84, 85, 86). The goal of the present study was to examine the structural and functional features of the CAP binding site from these ribosomes. Two approaches were employed in the examination of the CAP binding site and the results are reported in two parts. First, I report the initial examination of CAP analogs as inhibitors of the PTase activity of mitochondrial ribosomes in comparison to the inhibition of the PTase activity of bacterial ribosomes. This report includes the first usage of the CAP reduction intermediates and products as inhibitors of the PTase activities of bacterial and mitochondrial ribosomes. In addition, novel photo-activatable CAP analogs are introduced and tested in the PTase assay. A new structural and functional relationship of CAP and its ribosomal binding site was developed and is reported at the conclusion of the first half of the Results.

The second half of the Results reports the first use of the electrophilic CAP affinity analogs for the identification of proteins at the CAP binding site and within the domain of the Peptidyl Transferase Center of mammalian mitochondrial ribosomes.

Functional Studies of the Chloramphenicol Binding Site Through the Use of Chloramphenicol Analogs

One of the approaches employed to gain information about the functional features of the CAP binding site was through the use of analogs of chloramphenicol where specific changes have been made to the parent compound and the resultant changes in binding were then measured. In this manner, a functional understanding of the CAP binding site could be gained without having to dissect out the individual components of the ribosome. The constraints on chloramphenicol and its binding site could be determined, along with the types of interactions between CAP and its binding site.

Chloramphenicol was modified in either the dichloroacetate chain or the p-nitro moiety to produce the CAP analogs and the peptidyl transferase assay was employed to determine how well the analogs were bound to bacterial ribosomes and mammalian mitochondrial ribosomes.

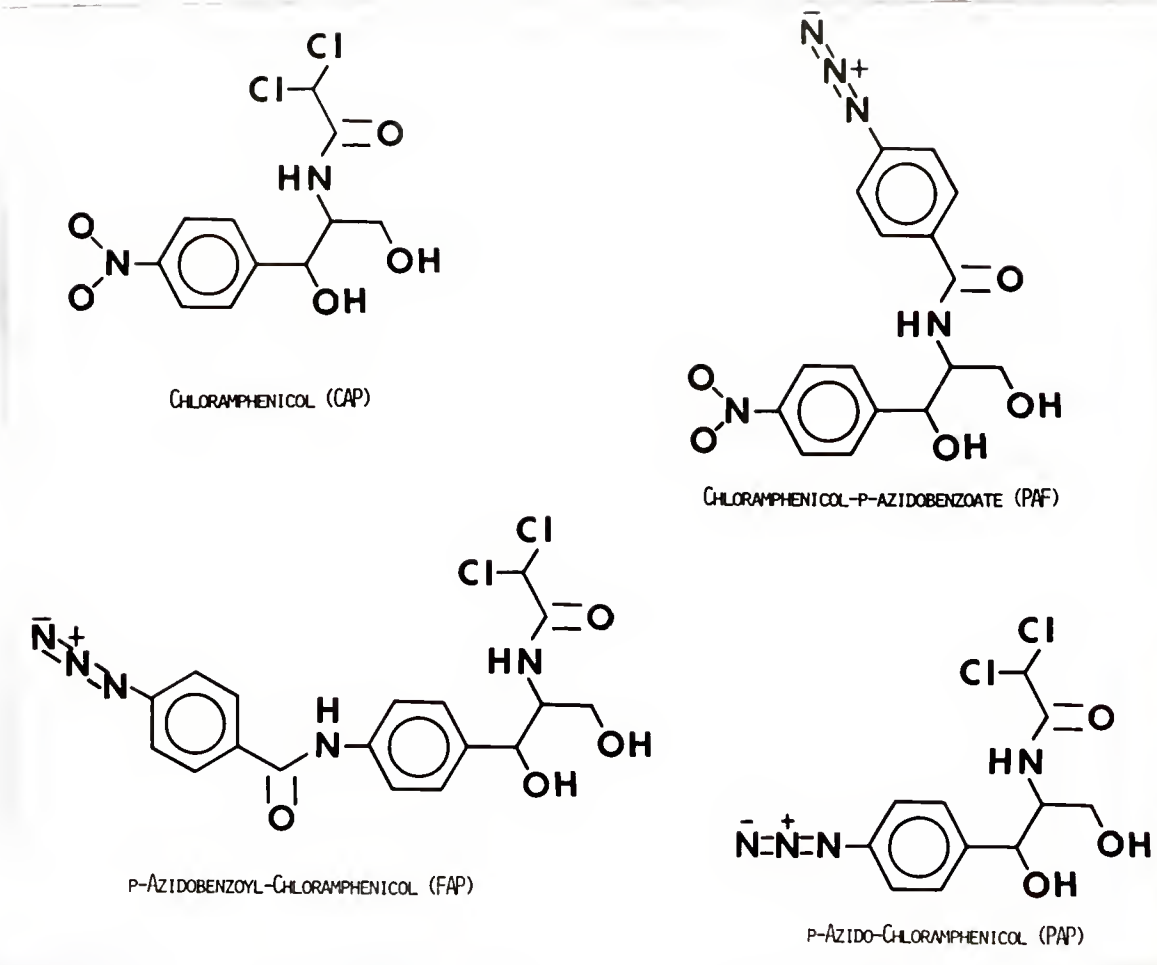
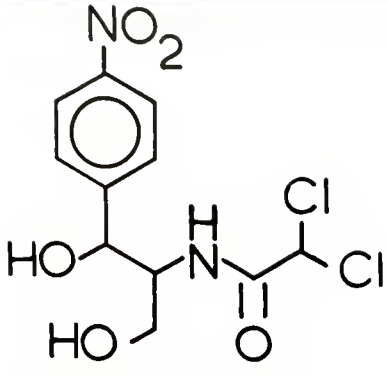
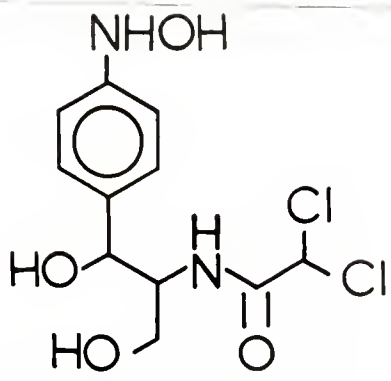


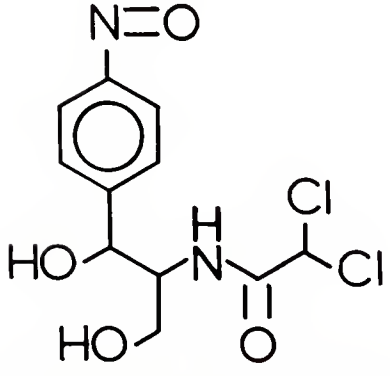
Figure 1. Comparison of the structures of chloramphenicol and the photo-activatable chloramphenicol analogs. (Hydrogens have been left off for clarity.) Notice that the phenylazido substituent of PAF is larger than the dichloroacetate of CAP.



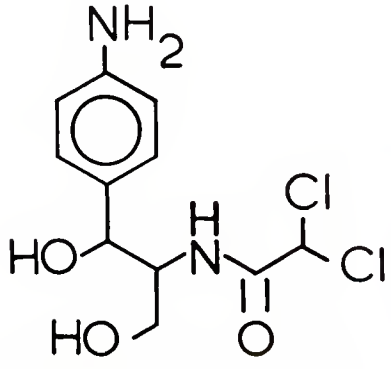
CHLORAMPHENICOL (CAP)



P-HYDROXYLAMINO CHLORAMPHENICOL (HACAP)



P-NITROSO CHLORAMPHENICOL (NOCAP)



P-AMINO CHLORAMPHENICOL (ACAP)

Figure 2. Comparison of the structures of chloramphenicol and the chloramphenicol reduction analogs. (Hydrogens have been left off for clarity.) Only the para position should bring about an alteration in the binding of these analogs since the rest of the molecule is left intact.

Chloramphenicol Analogs Modified in the Para-Nitro Moiety

CAP analogs with modifications in the para-nitro moiety belonged to two groups in this study. Two of the CAP analogs were potential photo-activatable affinity probes (Figure 1) by having aromatic azide substitutions onto CAP and the three other CAP analogs were the closely related family of nitro-reduction products (Figure 2), from chloramphenicol as the parent compound.

Chloramphenicol (CAP)

Mitochondrial ribosomes and bacterial ribosomes were subjected to CAP (0.01 mM to 1.0 mM) inhibition of their PTase activities. Details of the PTase assay are given in the Materials and Methods chapter. Briefly, the ribosomes are aliquoted (0.5 to 1.0 A_{260}) and the antibiotic is added at the proper concentration. The reaction is initiated by the addition of N-acetyl-[^{3}H]leucyl tRNA and after 10 minutes the reaction is terminated by the addition of 10 N KOH. The reaction mixture is neutralized with sodium phosphate buffer and then extracted with ethyl acetate. The ethyl acetate phase contains the N-acetyl [^{3}H]leucyl-puromycin adduct which is counted by LSC (see Materials and Methods). The control values are obtained from the puromycin-adduct formation by ribosomes in the absence of antibiotic. The data is presented as a percent of the untreated control value.

CAP inhibits the PTase activities of bacterial and mitochondrial ribosomes very well (Figure 3). Using the

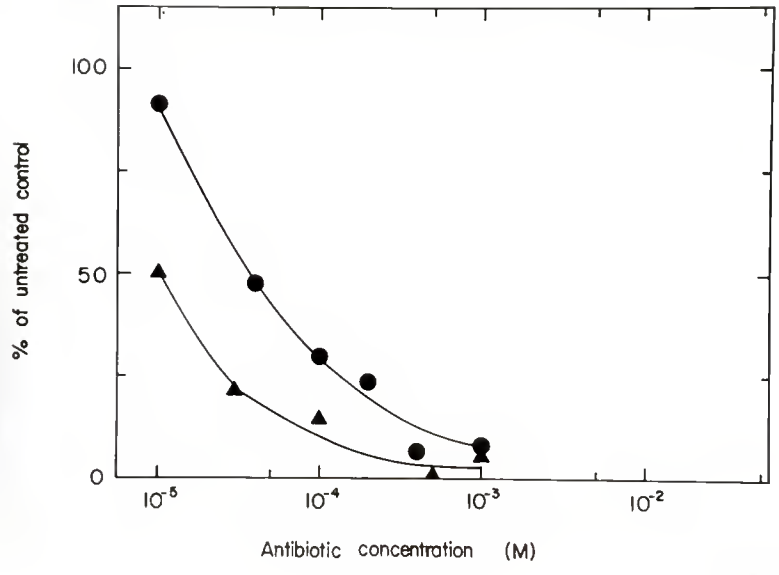


Figure 3. Comparison of the peptidyl transferase activities of mitochondrial ribosomes and bacterial ribosomes in the presence of chloramphenicol (CAP). Mitochondrial 39S subunits (●) and bacterial 50S subunits (▲) were treated with CAP at the indicated concentrations and assayed for their peptidyl transferase activities. The activities are reported as a percentage of the activities of ribosomes assayed in the absence of antibiotic. From Scatchard analysis of the data, a K'_d of 38 μM and 9.8 μM were calculated for the mitochondrial and bacterial ribosomes, respectively.

TABLE II

Apparent Dissociation Constants
for Chloramphenicol and
the Chloramphenicol Analogs

	K'_d (μM)	
	Ribosome Type	
Analog	Mitochondrial	Bacterial
CAP	38*	9.8
ACAP	362	103
FAP	970	860
HACAP	113	98
IAP	147	n.d.
NOCAP	190	83
PAF	n.a.	n.a.
PAP	n.a.	n.a.

*all values have units of μM

n.a. - not active

n.d. - not determined

The apparent dissociation constants were calculated using a Scatchard format as described in the Materials and Methods.

inhibition data transformed into the Scatchard format, an apparent dissociation constant (K'_d) for CAP of 9.8 μM was calculated for bacterial ribosomes and a K'_d of 38 μM, for mitochondrial ribosomes (Table II) (34). In general, bacterial ribosomes are more sensitive to peptidyl transferase inhibitors than mitochondrial ribosomes (34). This relationship holds true for chloramphenicol and the CAP analogs tested in the present study.

p-Amino-chloramphenicol (ACAP)

ACAP was the fully reduced analog of CAP where an amino group replaced the p-nitro moiety (Figure 2). The PTase activities with ACAP as an inhibitor were tested with concentrations of ACAP ranging from 0.01 mM to 5.0 mM (see Materials and Methods). ACAP was the weakest inhibitor of the CAP reduction products (Figure 4c and Table II), yet it remained an effective inhibitor of the PTase activities.

Soon after the discovery of chloramphenicol, it was suggested that ACAP would not inhibit protein synthesis in bacteria (87). This followed the mistaken notion that the bacterial nitro-reductase activity would detoxify CAP by reducing the nitro moiety to an inactive amine; however, ACAP itself had not been tested for activity by the investigators (87). As shown here, the reduced chloramphenicol did inhibit the PTase activities of both bacterial and mitochondrial ribosomes.

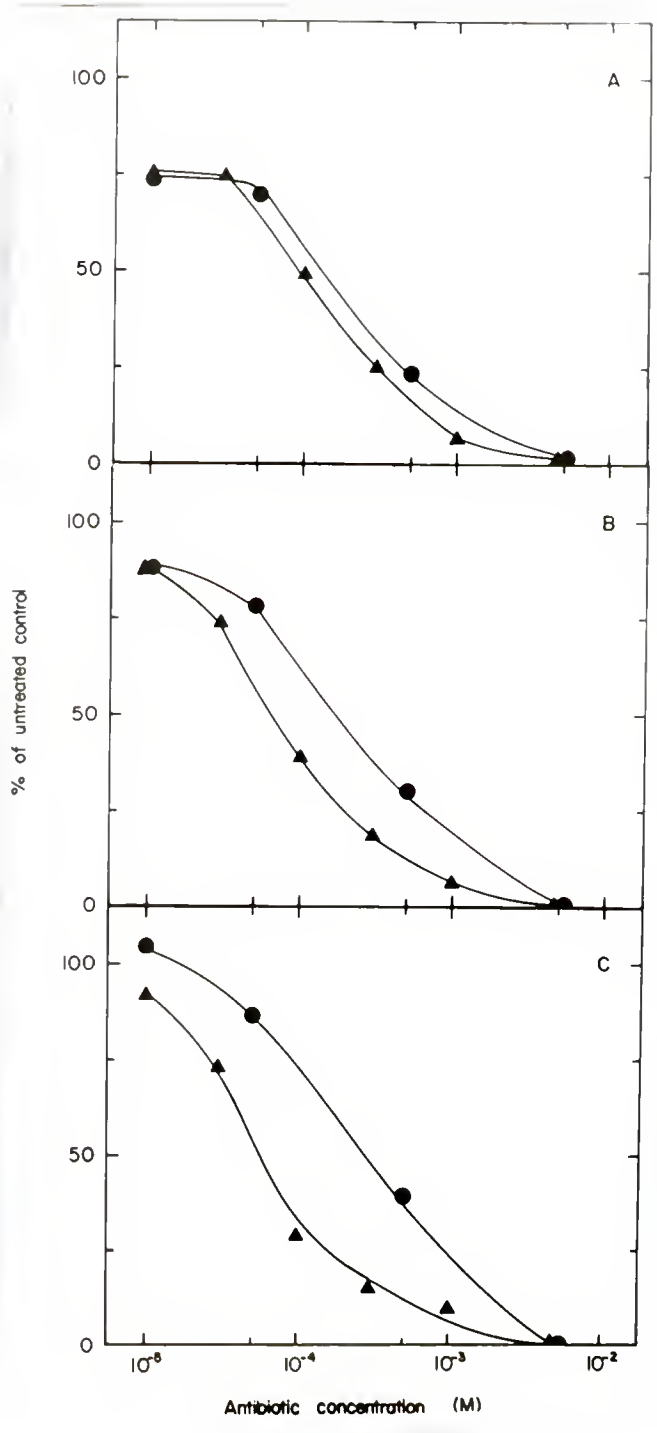


Figure 4. Comparison of the peptidyl transferase activities of mitochondrial ribosomes and bacterial ribosomes in the presence of the chloramphenicol reduction analogs. Mitochondrial 39S subunits (●) and bacterial 50S subunits (▲) were treated with p-hyroxylamino-chloramphenicol (HACAP) (A), p-nitroso-chloramphenicol (NOCAP) (B), or p-amino-chloramphenicol (ACAP) (C), at the indicated concentrations and assayed for their peptidyl transferase activities. The activities are reported as a percentage of the activities of ribosomes assayed in the absence of antibiotics.

p-Hydroxylamino-chloramphenicol (HACAP)

HACAP was a partially reduced CAP analog where the hydroxylamino group replaced the p-nitro moiety (Figure 2). HACAP was tested for the inhibition of the ribosomal PTase activities over the concentration range 0.01 mM to 5.0 mM (see Materials and Methods) and found to be a very effective inhibitor of the PTase activities of bacterial and mitochondrial ribosomes (Figure 4a). Although HACAP did not bind to ribosomes as well as CAP, it was the best CAP analog that I have synthesized in regard to its interaction with the mitochondrial ribosomes (Table II).

p-Nitroso-chloramphenicol (NOCAP)

NOCAP (Figure 2) was synthesized with a twofold purpose: 1) as a reduction product analog of CAP and a reversible inhibitor of the PTase activities of the bacterial and mitochondrial ribosomes and 2) as a potential electrophilic affinity probe for the CAP binding site.

NOCAP was used likewise from 0.01 mM to 5.0 mM to inhibit the PTase activities of the ribosomes (see Materials and Methods) and NOCAP was found to be a good inhibitor of the PTase activities of both the bacterial ribosomes and the mitochondrial ribosomes (Figure 4b and Table II). With the criterion of "interaction" with the ribosome met, the continued testing of NOCAP for use as an affinity probe was warranted. (The use of NOCAP as an affinity probe is discussed below.)

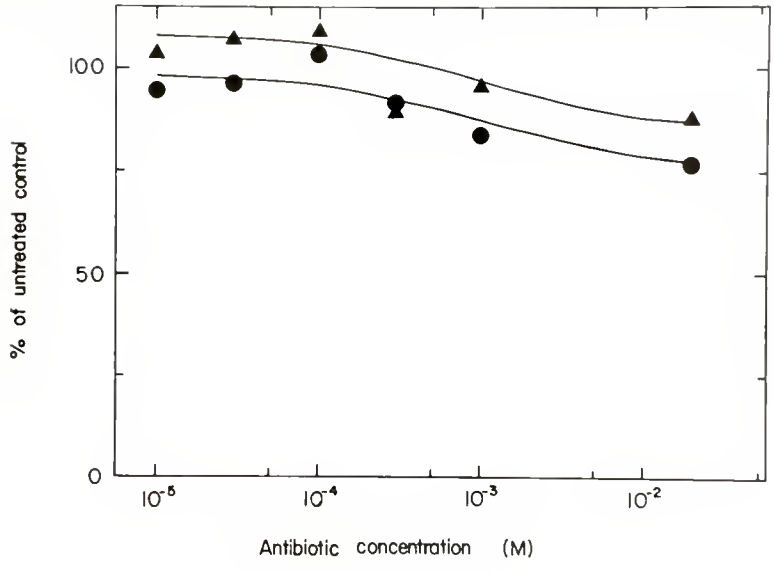


Figure 5. Comparison of the peptidyl transferase activities of mitochondrial ribosomes and bacterial ribosomes in the presence of p-azido-chloramphenicol (PAP). Mitochondrial 39S subunits (●) and bacterial 50S subunits (▲) were treated with PAP at the indicated concentrations and assayed for their peptidyl transferase activities. The activities are reported as a percentage of the activities of ribosomes assayed in the absence of antibiotic. From the lack of effect on the peptidyl transferase activity, PAP was assigned a biological activity of zero for the structure-activity analysis.

p-Azido-chloramphenicol (PAP)

PAP (Figure 1) had previously been synthesized for use as a photo-affinity probe for the CAP binding site of bacterial ribosomes (64, 65). The authors presented data which suggested that there was some inhibitory activity towards the PTase Center of bacterial ribosomes (65). In the present study, PAP was found to provide no inhibition of the PTase activities of bacterial or mitochondrial ribosomes when tested over a broad concentration range (0.01 mM to 18.5 mM) (Figure 5). (The PTase assay was conducted in the dark to prevent photo-activation of the azido function of PAP, as described in the Materials and Methods.)

Since PAP does not bind to either ribosome, it was removed from consideration as an affinity probe and was assigned a biological activity of zero for the structure-activity relationship to be discussed below.

p-Azidobenzoamido-chloramphenicol (FAP)

FAP was the second of the photo-activatable CAP analogs modified in the p-nitro position (Figure 1). (The PTase assay involving FAP was also carried out in the dark, see Materials and Methods.) The added phenylazide provided the photo-activatable nucleus, but did not prevent FAP from inhibiting the PTase activities of the ribosomes (Figure 6 and Table II). It had been shown that CAP could accept bulky substituents from the p-nitro position without losing its ability to inhibit the PTase activities of bacterial

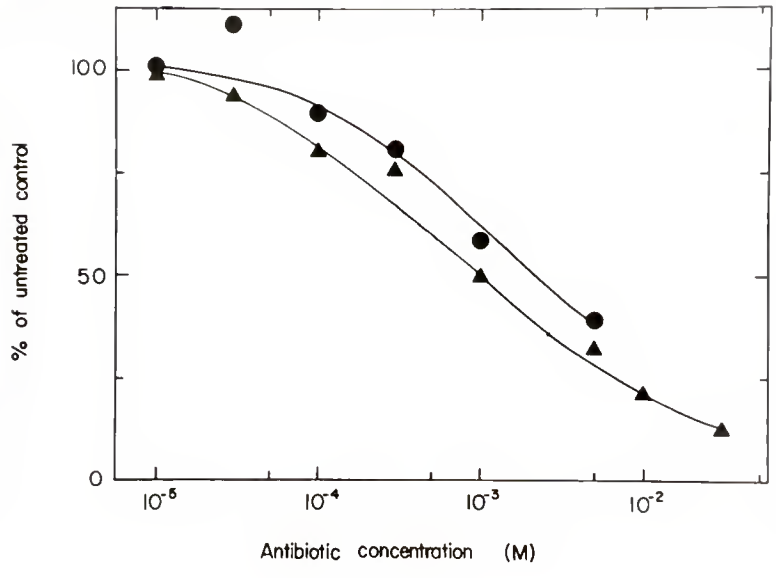


Figure 6. Comparison of the peptidyl transferase activities of mitochondrial ribosomes and bacterial ribosomes in the presence of p-azidobenzoamido-chloramphenicol (FAP). Mitochondrial 39S subunits (●) and bacterial 50S subunits (▲) were treated with FAP at the indicated concentrations and assayed for their peptidyl transferase activities. The activities are reported as a percentage of the activities of ribosomes assayed in the absence of antibiotic.

ribosomes. With this fact in mind, FAP had been synthesized as a novel photo-affinity analog of CAP.

Chloramphenicol Analogs Modified in the Dichloroacetate Chain

Idoamphenicol (IAP)

Iodoamphenicol (Figure 7) had previously been used as an affinity probe for the CAP binding site in bacterial ribosomes (59, 61). IAP was therefore selected for use as an affinity probe for mammalian mitochondrial ribosomes. (Details of the affinity labelling experiments to be discussed below.) To be useful as an affinity probe, an antibiotic must be shown to interact with the ribosomes. IAP (0.01 mM to 5.0 mM) was allowed to inhibit the PTase activity of the mitochondrial ribosomes under conditions where irreversible inactivation of the Ptase activity was thought to be negligible (see Materials and Methods).

IAP is an effective reversible inhibitor of the PTase activity of the mitochondrial ribosomes (Figure 8). From Scatchard analysis of the inhibition data, IAP has a K'_d of 147 μM where CAP has a K'_d of 38 μM.

Chloramphenicol-p-azidobenzoate (PAF)

PAF (Figure 1) with p-azidobenzoate substituting for the dichloroacetate, was synthesized as a potential photo-activatable affinity probe for the CAP binding site. PAF has been independently synthesized and tested for the inhibition of the PTase activity of bacterial ribosomes by another group of investigators and their data presented

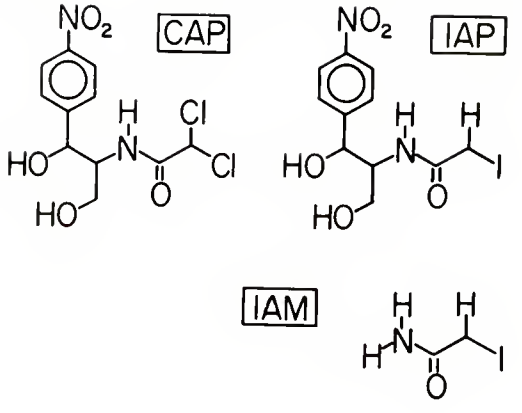


Figure 7. Comparison of the structures of chloramphenicol, iodoamphenicol, and iodoacetamide. Chloramphenicol (CAP), iodoamphenicol (IAP), and iodoacetamide (IAM) are compared, where IAP has an iodoacetate instead of the dichloroacetate. IAP can be considered a substituted IAM, both contain the reactive alkyl halide, but IAM does not contain the active site directing p-nitrophenylpropanediol moiety.

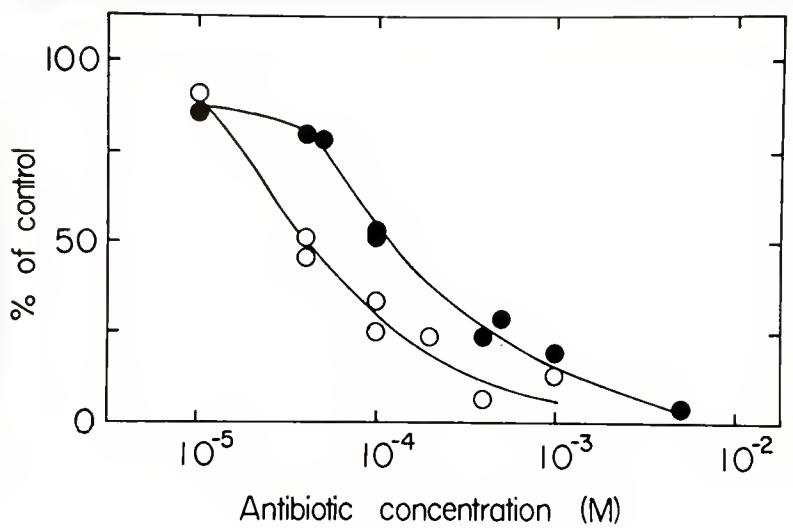


Figure 8. Comparison of the peptidyl transferase activities of mitochondrial ribosomes in the presence of chloramphenicol (CAP) or iodoamphenicol (IAP). Mitochondrial 39S subunits were treated with CAP (○) or IAP (●) at the indicated concentrations and assayed for their peptidyl transferase activities. The activities are reported as a percentage of the activities of ribosomes assayed in the absence of antibiotic.

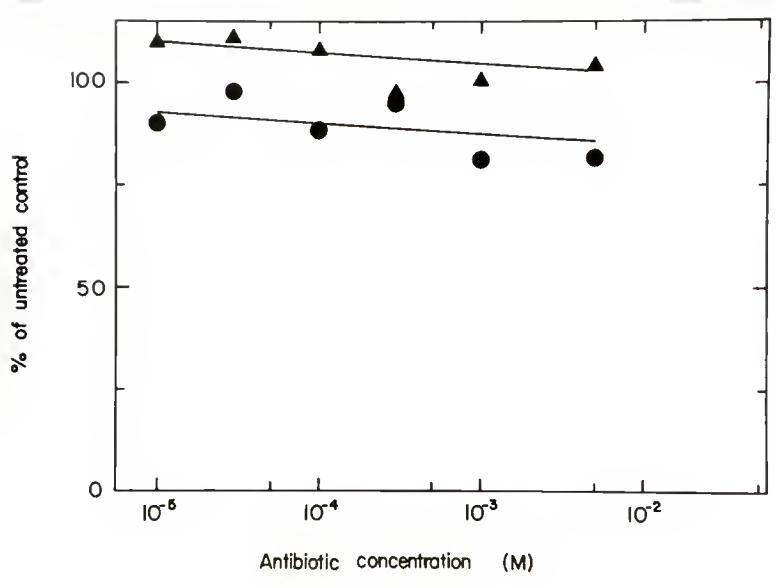


Figure 9. Comparison of the peptidyl transferase activities of mitochondrial ribosomes and bacterial ribosomes in the presence of chloramphenicol-p-azidobenzoate (PAF). Mitochondrial 39S subunits (●) and bacterial 50S subunits (▲) were treated with PAF at the indicated concentrations and assayed for their peptidyl transferase activities. The activities are reported as a percentage of the activities of ribosomes assayed in the absence of antibiotic. From the lack of effect on the peptidyl transferase activities, PAF was assigned a biological activity of zero for the structure-activity analysis.

little promise that PAF would be a useful CAP analog (88). In the present study, PAF was tested for its ability to inhibit the PTase activities of mitochondrial and bacterial ribosomes over a range of concentrations from 0.01 mM to 5.0 mM (the assay was performed in the dark, see Materials and Methods). PAF was found not to inhibit the PTase activities of either kind of ribosome (Figure 9); consequently PAF was discarded from consideration as an affinity probe and was assigned a biological activity of zero for the structure-activity relationships (discussed below).

Structure-Activity Relationships of Chloramphenicol Analogs

Two major concepts were developed concerning the CAP binding site of bacterial ribosomes and mitochondrial ribosomes, involving two domains of the CAP molecule, the dichloroacetate chain and the para-nitro moiety. The dichloro group has a size constraint, so that an analog such as IAP is still capable of binding to the CAP site, since the iodo substitution has a smaller size than the dichloro moiety. On the other hand, PAF cannot, since the phenylazido substitution has a much larger bulk than the dichloro group of CAP.

Using bacterial growth as an indication of the inhibitory activity of CAP analogs, it has been reported previously that the molar volume and electronegativity of the dichloro position are important for the binding of CAP to the CAP binding site (35, 36, 37, 89, 90, 91). Even

though only two CAP analogs with dichloroacetate chain substitutions were tested in this study, it is apparent from these data that the molar volume of this position is an important determinant as to whether the CAP analog will bind to mitochondrial ribosomes, as well.

Several studies have examined CAP analogs with p-nitro substitutions and the resultant inhibition of bacterial growth as an indicator of the binding properties of the analogs. From the studies, the electronegative property of the para position had been suggested to be the major contribution of the p-nitro moiety for the binding of CAP to the CAP binding site of bacterial ribosomes (36, 38, 39, 91).

In the present study, a "family" of closely related CAP analogs, the nitro reduction products and PAP, have been tested for their abilities to bind to bacterial and mitochondrial ribosomes. To examine the relationship of the electronegativity of the para position to the ability of the CAP analogs to bind to the ribosomes, the Hammett sigma value, (as a measure of electronegativity), was plotted versus the activity of the CAP analog as measured in the PTase assay. A poor correlation was observed (Figure 10). ACAP and HACAP are much more active than their respective electronegativities would predict, whereas PAP, which is inactive, would be predicted to bind to the ribosomes about as well as NOCAP.

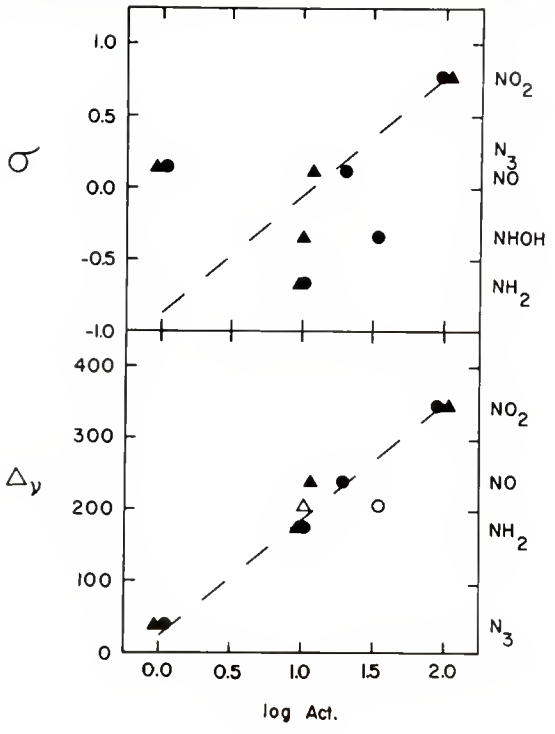


Figure 10. Structure-activity relationships of chloramphenicol analogs with para-nitro substitutions. In the upper panel is plotted the Hammett sigma value (σ) to activity relationship, which exhibits a low correlation. In the lower panel is plotted the shift in infrared aborbance frequency ($\Delta\nu$), as a measure of hydrogen bond strength, to activity, which exhibits a high degree of correlation. The physical parameters were found in (71, 72, 73) and the activities for the mitochondrial ribosomes (●) and bacterial ribosomes (▲) were determined as reported in the Materials and Methods. The para substituents are listed on the right; NO_2 (CAP), N_3 (PAP), NO (NOCAP), NHOH (HACAP), and NH_2 (ACAP). The dashed lines represent a direct correction of the physical parameters of the para positions to the activity of the antibiotic analog. The open circle and open triangle represent the hydrogen bond strength to activity relationship of HACAP for mitochondrial and bacterial ribosomes, respectively.

Besides being extremely electronegative, another striking property of nitro groups is their ability to form hydrogen bonds (H-bonds) with suitable donors (72, 73, 92). Using the shift in the infrared absorbance frequency of donor hydroxyl groups forming hydrogen bonds with the para substituents, a good correlation between the H-bond strength and the inhibitory activities of the CAP analogs was found (Figure 10). PAP, with the azido substitution, binds poorly to the ribosomes and is a poor H-bond acceptor (72, 73, 92).

Electronegativity has been loosely correlated with hydrogen bond strength, but features such as the directionality of the H-bond are not involved in the correlation (92). The previous suggestion of the para-nitro position requiring a high electronegativity for good CAP analog binding may have been because the substituents used had a high degree of correlation between the H-bond strengths and the electronegativities.

I propose that the essential feature of the para-nitro position of CAP is its ability to act as a hydrogen bond acceptor to a hydrogen bond donor in the CAP binding site, and that this arrangement plays a role in the anchoring of CAP to the CAP binding site of bacterial ribosomes and mitochondrial ribosomes.

Affinity Labelling for Structural (and Functional?) Proteins of the Peptidyl Transferase Center and the Chloramphenicol Binding Site of Mammalian Mitochondrial Ribosomes

Affinity labelling is the technique with the highest potential for allowing the identification of structurally important ribosomal proteins in the CAP binding site, which, in addition, may be functionally important for the peptidyl transferase activity, as well as for the binding of CAP. Verification of the functional importance of the affinity labelled proteins can be obtained through the use of other techniques such as salt stripping and functional reconstitution (40, 41, 42).

Two electrophilic CAP analogs were used in this study to probe the CAP binding site by affinity labelling the ribosomal proteins therein. Iodoamphenicol was the dichloroacetate substituted analog of CAP and p-nitroso-chloramphenicol was the p-nitro substituted analog of CAP used, where the distal positions of the parent molecular structure of CAP have the electrophilic modifications. In this manner, it was expected that proteins associated with the binding of the distal portions of CAP might be affinity labelled, if indeed more than one protein comprises the CAP binding site, and if the proteins contain the appropriate nucleophiles.

Iodoamphenicol as the Affinity Probe

Iodoamphenicol was among the first affinity labelling reagents used to probe for proteins of the CAP binding site of bacterial ribosomes (59, 61). Therefore, IAP was selected to be used to affinity label proteins from the CAP binding site of mammalian mitochondrial ribosomes. Dr. Olaf Pongs had donated a small sample of unlabelled IAP to our laboratory for the initial examination of the utility of IAP as an affinity probe for the mitochondrial ribosomes. I synthesized a fresh stock of unlabelled IAP (see Materials and Methods) to complete the tests of the interaction of IAP with the mitochondrial ribosomes. For the affinity labelling experiments, I synthesized [^{3}H]IAP from [^{3}H]iodoacetic acid and CAP base as the starting materials (see Materials and Methods).

Effect of iodoamphenicol on the peptidyl transferase activity of mitochondrial ribosomes

Reversible effect. Iodoamphenicol reversibly inhibits the PTase activity of mitochondrial ribosomes in a manner similar to CAP (Figure 8). The interaction of IAP (K'_d 147 μM) is about fourfold lower than the interaction of CAP (K'_d 38 μM) with the mitochondrial ribosomes. Bacterial ribosomes have the same relationship of a fourfold difference, with K'_d's for IAP and CAP of 15 μM and 4 μM, respectively (59).

Irreversible effect. Since IAP binds reversibly to the mitochondrial ribosomes, it was expected that a

prolonged incubation of the ribosomes in the presence of IAP would allow IAP to covalently attach to the CAP binding site of the ribosomes and thereby irreversibly inhibit the PTase activity associated with the ribosomes. It had been determined in our laboratory that an incubation of the mitochondrial ribosomes with iodoamphenicol would bring about some irreversible inactivation of the PTase activity of these ribosomes (Mary Conde, personal communication). The irreversible inactivation of the PTase activity saturates at a plateau of about 25% (Figure 11).

The level of irreversible inactivation of the PTase activity of mitochondrial ribosomes is lower than that observed when IAP was used to irreversibly inhibit bacterial ribosomes (25% and 75% respectively) (59, 61). This seemingly low level of irreversible inhibition can be interpreted to mean that the amino acid nucleophile being alkylated by IAP may not be precisely oriented within the CAP binding site to allow the covalently attached IAP molecule to completely obscure the site and thereby prevent the PTase activity. The CAP binding site can be thought of as being occupied 25% of the time or as being 25% blocked when IAP has been covalently attached to the CAP binding site.

Iodoamphenicol can be thought of as being an N-substituted IAM (Figure 7). IAP and IAM would react with the same proteins, but IAM lacks the necessary active-site-directing p-nitrophenyl-1,3-propanediol moieties.

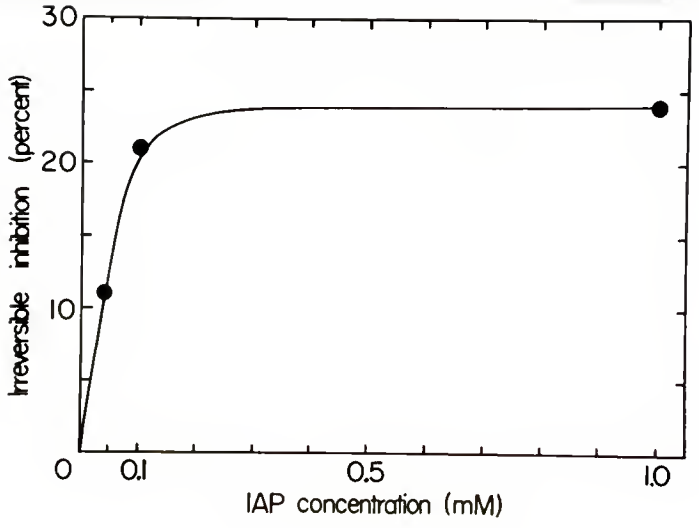


Figure 11. Peptidyl transferase activity of mitochondrial ribosomes after prolonged incubations with iodoamphenicol (IAP). Mitochondrial ribosomes were incubated with IAP at the indicated concentrations for one hour at 34°C, dialyzed extensively overnight, centrifuged 100,000 xg through a 20% sucrose wedge, and then assayed for their peptidyl transferase activity. A saturation of the inactivation is observed for concentrations above 0.1 mM IAP at a level of about 25%. (Data from Mary Conde)

Under the conditions where IAP could irreversibly inhibit the mitochondrial ribosomal PTase activity, IAM was found not to inactivate the ribosomes and CAP was binding in a fully reversible manner (Mary Conde, personal communication) (Table III).

The irreversible inactivation observed for mitochondrial ribosomes after incubation with IAP is probably not simply due to the alkylation of an essential amino acid in the PTase Center (since IAM does not cause inactivation), but rather that the covalently attached IAP, because of it's extra bulk, is able to prevent the binding of the substrates required for the PTase activity, in the region of the CAP binding site.

Through the reversible binding of IAP to the CAP binding site of mitochondrial ribosomes and the irreversible inactivation of the PTase activity, a functional interaction with the ribosomes had been established. The next step was to use radiolabelled IAP as an affinity probe for proteins of the CAP binding site of mitochondrial ribosomes.

1-D PAGE analyses of [^{3}H]iodoamphenicol labelling of ribosomal large subunit proteins

[^{3}H]Iodoamphenicol was synthesized from chloramphenicol base and [^{3}H]iodoacetic acid as described in the Materials and Methods. The [^{3}H]IAP was judged to be greater than 99% pure from TLC analyses (see Materials and Methods).

TABLE III

Peptidyl Transferase Activity of Mitochondrial Ribosomes Treated with Iodoamphenicol, Chloramphenicol or Iodoacetamide

Agent Present During Incubation	Percent of Control Activity	
	Before Dialysis	After Dialysis
IAP (1 mM)	19	76
CAP (1 mM)	13	93
IAM (4 mM)	98	96

Mitochondrial ribosomes were incubated with the reagent for one hour at 34°C, dialyzed overnight, and concentrated by centrifugation. The activities were determined using the peptidyl transferase assay and are reported as a percentage of the activity of ribosomes incubated in the absence of reagent. (Data from Mary Conde)

The affinity labelling conditions were selected from the consideration of several criteria. The buffer was developed to be compatible with the mitochondrial ribosomal subunits (200 mM KCl and 10 mM Mg Acetate) and to be free from nucleophilic components that would compete with the affinity labelling reaction (triethanolamine instead of Tris and no 2-mercaptoethanol). (Buffer L: 200 mM KCl, 10 mM Mg Acetate, 25 mM triethanolamine HCl, pH 7.5). The ribosomal concentration was chosen to be approximately 1 A_{260}/10 µl, which is 5.5 µM for the 39S large ribosomal subunit. This provides for a concentrated ribosomal solution with a small volume to allow less of the radiolabelled probe to be used for each experiment so that several experiments could be accomplished. The concentrations of [^{3}H]IAP selected for the affinity labelling experiments extends through the response range of IAP with the ribosomes as determined from the reversible and irreversible PTase assays, and provides for [^{3}H]IAP to ribosome ratios of approximately 1 to 1 through 200 to 1.

Mitochondrial ribosomal large subunits were incubated for one hour at 34°C with [^{3}H]IAP at concentrations of a) 0.005 mM, b) 0.01 mM, c) 0.05 mM, d) 0.1 mM, e) 0.5 mM, and f) 1.0 mM. Subsequently, the ribosomal proteins were extracted and separated by one-dimensional SDS PAGE (see Materials and Methods). One protein migrating at Band 4 in the SDS unidimensional gels (Figure 12, panel A, stained electropherogram) labels at low concentrations of

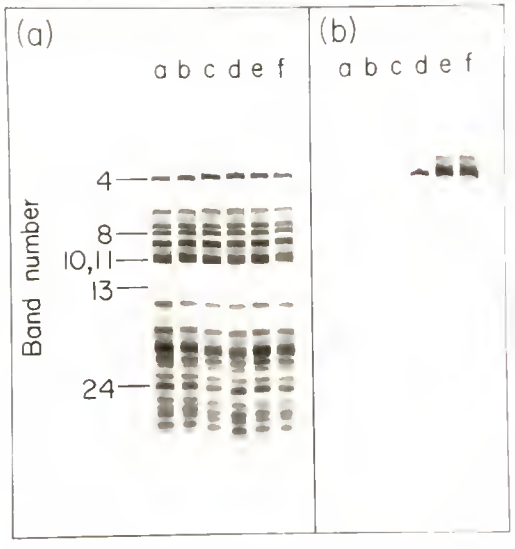


Figure 12. One-dimensional PAGE analyses of the [^{3}H]iodoamphenicol ([^{3}H]IAP) labelling of mitochondrial large subunit proteins. Mitochondrial 39S subunits were incubated for one hour at 34°C with [^{3}H]IAP at (a) 0.005 mM, (b) 0.01 mM, (c) 0.05 mM, (d) 0.1 mM, (e) 0.5 mM, and (f) 1.0 mM. The ribosomal proteins were extracted and separated by 1-D PAGE as shown in the electropherogram (panel A). The fluorogram (four day exposure) of the dried gel is shown in panel B and from its inspection, a protein in Band 4 can be observed to label at low concentrations of probe and be the most intensely labelled protein at higher concentrations.

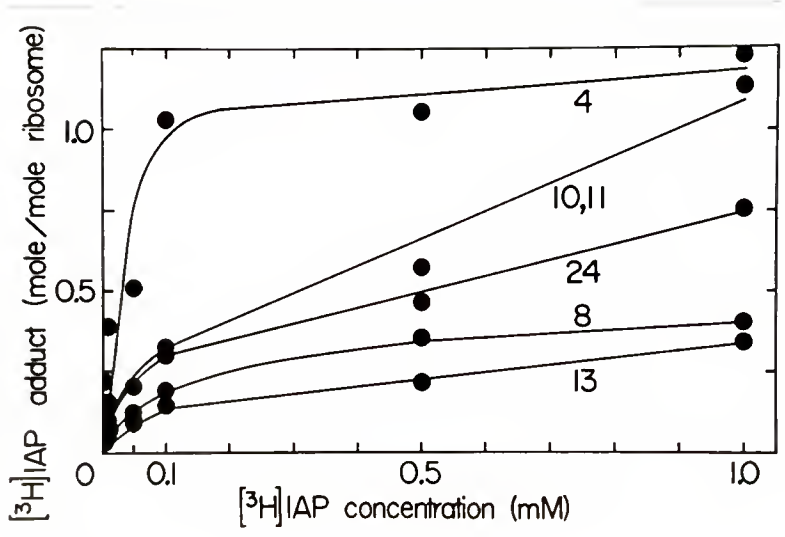


Figure 13. Comparison of label incorporated into individual protein bands of [^{3}H]iodoamphenicol ([^{3}H]IAP) labelled mitochondrial ribosomal large subunit proteins separated by 1-D PAGE. Individual protein bands were cut from the dried gel (shown in Figure 12), digested, and their label content was determined by liquid scintillation counting. Only Band 4 exhibits a labelling profile which saturates at a stoichiometric level of [^{3}H]IAP incorporation into a protein. The saturation effect is observed for concentrations of [^{3}H]IAP above 0.1 mM, which parallels the saturation of irreversible inactivation observed previously (Figure 11).

[^{3}H]IAP (Figure 12, panel B, fluorogram), where label does not appear in the other protein bands. After fluorography, the protein bands from the stained gel were cut, digested, and counted by liquid scintillation counting (see Materials and Methods). As was observed in the fluorogram (Figure 12, panel B),the protein in Band 4 has the greatest amount of label at the low concentrations and the labelling pattern saturates at one mole [^{3}H]IAP-adduct per mole of ribosome at 0.1 mM [^{3}H]IAP (Figure 13). The other ribosomal proteins do not label well at low concentrations of [^{3}H]IAP (no visible bands by fluorography, Figure 12, panel B), and as shown in Figure 13, the labelling patterns of some of these ribosomal proteins approach stoichiometry in a non-saturating manner only at a high [^{3}H]IAP concentration (1.0 mM). The appearance of label on a ribosomal protein at low [^{3}H]IAP concentrations and the saturation of the labelling pattern on this protein at high [^{3}H]IAP concentrations, suggest that this ribosomal protein was affinity labelled. Band 4 has a molecular weight of approximately 45,000 (see Materials and Methods) and was tentatively assigned as ribosomal protein L2.

1-D PAGE analyses of [^{14}C]iodoacetamide labelling of ribosomal large subunit proteins

Lacking the CAP binding site directing groups, [^{14}C]IAM should produce a different labelling pattern on the [^{3}H]IAP affinity labelled ribosomal proteins. The

[^{3}H]IAP affinity labelled proteins would be expected to label less well with [^{14}C]IAM.

Mitochondrial ribosomes were incubated with [^{14}C]IAM under conditions similar to those used for the incubation of [^{3}H]IAP with the ribosomes (see Materials and Methods). The ribosomal proteins were extracted and separated by unidimensional SDS PAGE (Figure 14, panel A). Fluorography of the dried gel (see Materials and Methods), revealed a labelling pattern that was different from the [^{3}H]IAP labelling of the ribosomal proteins at low concentrations of labelling reagent (Figure 14, panel B), although at high concentrations of [^{14}C]IAM and [^{3}H]IAP the labelling patterns appeared to be more similar (compare Figure 12, panel B to Figure 14, panel B). The protein bands of the dried gel were cut, digested, and counted by LSC (Figure 15) (see Materials and Methods), as had been the bands from the IAP gel. In contrast to the labelling of ribosomal proteins by [^{3}H]IAP, [^{14}C]IAM labels all the ribosomal proteins in a strictly concentration-dependent manner (Figure 13 compared to Figure 15). The saturation of labelling on a protein is not observed when [^{14}C]IAM labels the ribosomes and stoichiometric labelling occurs only at a high concentration of labelling reagent (Figure 15). Several of the protein bands label similarly whether [^{3}H]IAP or [^{14}C]IAM is used as the labelling reagent (compare Bands 10, 11, 8 and 13 from Figures 13 and 15). Of special interest is the fact

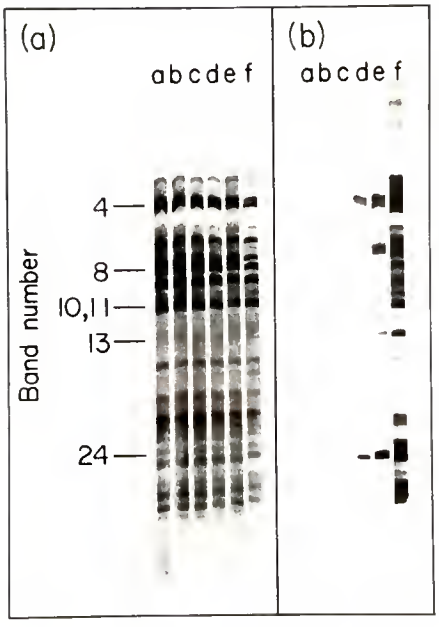


Figure 14. One-dimensional PAGE analyses of the [^{14}C]iodoacetamide ([^{14}C]IAM) labelling of mitochondrial ribosomal large subunit proteins. Mitochondrial 39S subunits were incubated for one hour at 34°C with [^{14}C]IAM at (a) 0.004 mM, (b) 0.01 mM, (c) 0.04 mM, (d) 0.1 mM, (e) 0.4 mM, and (f) 1.0 mM. The ribosomal proteins were extracted and separated by 1-D PAGE as shown in the electropherogram (panel A). The fluorogram (four day exposure) of the dried gel is shown in panel B, where several protein bands show the incorporation of label at high concentrations, but none exhibit labelling at a low concentration of [^{14}C]IAM.

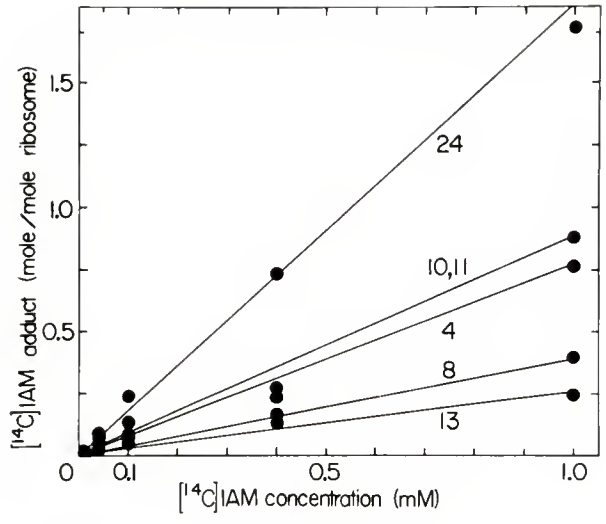


Figure 15. Comparison of label incorporated into individual protein bands of [^{14}C]iodoacetamide ([^{14}C]IAM) labelled mitochondrial ribosomal large subunit proteins separated by 1-D PAGE. Individual protein bands were cut from the dried gel (shown in Figure 14), digested, and their label content was determined by liquid scintillation counting. None of the protein bands exhibit a saturating labelling profile. Some of the bands are only approaching stoichiometric labelling at 1.0 mM [^{14}C]IAM. The labelling profile observed for Band 4 appears to be only concentration-dependent, like that observed for the other bands, in contrast to its labelling with [^{3}H]IAP.

that the ribosomal protein in Band 4 labels like other ribosomal proteins in a non-saturating manner with [^{14}C]IAM, in contrast to its [^{3}H]IAP labelling profile (Figures 13 and 15).

The ribosomal protein in Band 4 appears to be specifically affinity labelled by the CAP affinity analog, [^{3}H]IAP, when comparing the labelling patterns of ribosomal proteins with [^{3}H]IAP and [^{14}C]IAM.

Effect of chloramphenicol on the labelling of ribosomal large subunit proteins by [^{3}H]iodoamphenicol

Chloramphenicol was used to competitively block the labelling reaction of [^{3}H]IAP with the mitochondrial ribosomes. It was expected that CAP could effectively reduce the amount of [^{3}H]IAP that would be available to bind to the CAP binding site and thereby reduce the amount of [^{3}H]IAP-adduct formation with ribosomal proteins at the CAP binding site.

The mitochondrial ribosomal large subunits were incubated in the presence of 0.1 mM [^{3}H]IAP and 4 mM CAP, to act as a competitive blocker of the labelling reaction, or the subunits were incubated only with 0.1 mM [^{3}H]IAP (see Materials and Methods). The ribosomal proteins were extracted and separated by unidimensional SDS PAGE, after which the stained protein bands were cut, digested, and counted by LSC (see Materials and Methods). There were 33 resolved bands cut for LSC (Figure 16, A). As can be observed from a histogram of the results (Figure 16, B),

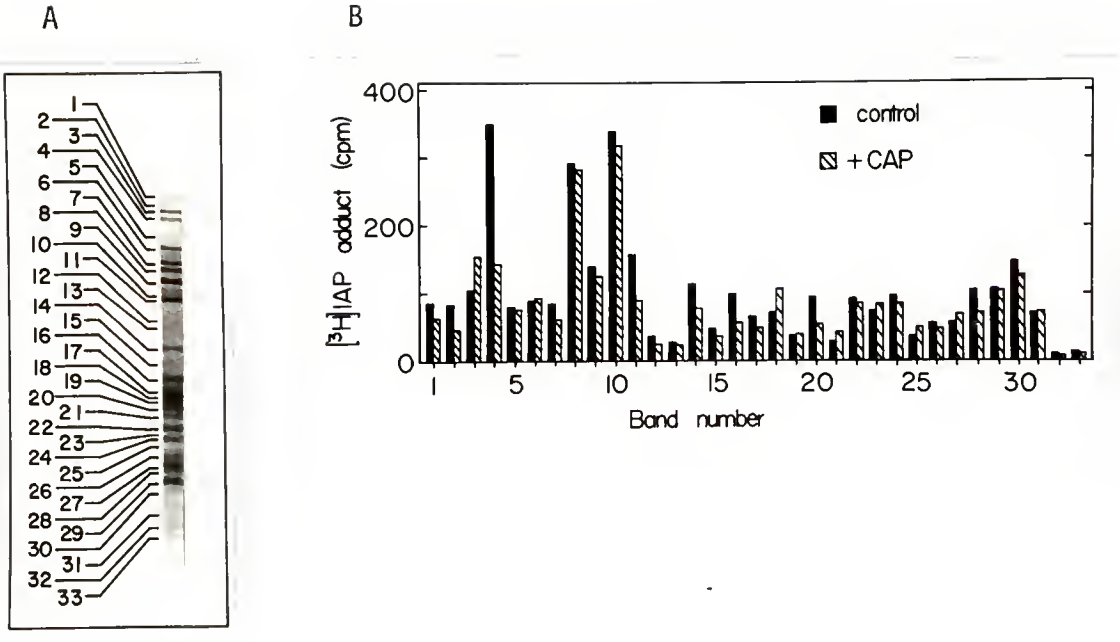


Figure 16. Comparison of label incorporated into individual protein bands separated by 1-D PAGE, from mitochondrial ribosomal large subunits labelled by [^{3}H]iodoamphenicol ([^{3}H]IAP) in the absence and presence of chloramphenicol (CAP). Mitochondrial 39S subunits were incubated with 0.1 mM [^{3}H]IAP in the absence and presence of 4.0 mM CAP for one hour at 34°C. The ribosomal proteins were extracted and separated by 1-D PAGE, where there were 33 resolvable bands (A). The bands of the two gels were cut, digested, and counted by liquid scintillation counting in parallel. As can be observed from the histogram (B), only Band 4 labels well with [^{3}H]IAP (filled boxes) and has the extent of labelling reduced to the level observed for the other proteins in the presence of CAP (slashed boxes). Absence of CAP, filled boxes and presence of CAP, slashed boxes.

only one protein band labels well with [^{3}H]IAP and has the amount of label greatly reduced (by 60%) when the competitive blocker, CAP is present (Figure 16, B). This Band 4 protein, tentatively named L2 of the mitochondrial ribosomal large subunit, was assigned to the region of the CAP binding site of these ribosomes on the basis of the labelling patterns observed with [^{3}H]IAP and [^{14}C]IAM, and by the CAP competition of the [^{3}H]IAP labelling of this protein.

Effect of chloramphenicol on the labelling of ribosomal proteins by [^{14}C]iodoacetamide

To detect if the binding of CAP to the ribosomes induces a structural change, which alters the accessibility of the nucleophile in Band 4 to labelling reagents, mitochondrial ribosomal large subunits were incubated with [^{14}C]IAM in the presence and absence of CAP (see Materials and Methods). Following the incubation, the ribosomal proteins were extracted, separated by unidimensional SDS PAGE, and the gels were prepared for fluorography (see Materials and Methods). From the densitometric scans of the fluorograms (see Materials and Methods) (Figure 17), virtually no difference was observed in the [^{14}C]IAM labelling pattern of ribosomal proteins in the presence or absence of CAP. In particular, the region of Band 4 is identical whether CAP was present to block the labelling reaction or was not present.

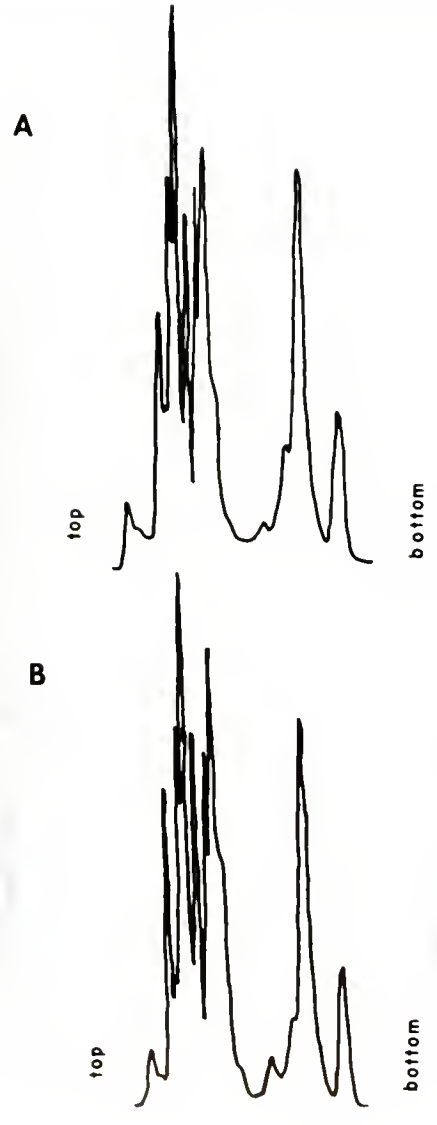


Figure 17. Comparison of the tracings of densitometric scans from the fluorograms (16 day exposure) of 1-D PAGE separated mitochondrial ribosomal large subunit proteins labelled with [^{14}C]iodoacetamide ([^{14}C]IAM), where chloramphenicol (CAP) was included or not included in the labelling reactions. Mitochondrial 39S subunits were incubated for one hour at 34°C with 1.0 mM [^{14}C]IAM in the absence (A) and presence (B) of 4.0 mM CAP. The ribosomal proteins were extracted, separated by 1-D PAGE, and analyzed by fluorography. The fluorograms were densitometrically scanned (the top of the gel is on the left and the bottom on the right), revealing that CAP does not exert an effect on the [^{14}C]IAM labelling of ribosomal proteins. This is in contrast to the [^{3}H]IAP labelling of ribosomal proteins, suggesting that CAP can block the specific affinity labelling by [^{3}H]IAP.

It can be concluded that the CAP competition of the [^{3}H]IAP labelling of ribosomal proteins is not an artifact of CAP induced conformational alterations of proteins at the CAP binding site.

2-D PAGE analyses of [^{3}H]iodoamphenicol labelling of ribosomal large subunit proteins

Two-dimensional PAGE was used for conclusive identification of the ribosomal protein that was specifically labelled with [^{3}H]IAP. Mitochondrial ribosomal large subunits were incubated with 0.01 mM or 0.1 mM [^{3}H]IAP in the absence and presence of 4 mM CAP (see Materials and Methods). The ribosomal proteins were extracted and separated by 2-D PAGE (see Material and Methods). The polyacrylamide gels were analyzed by fluorography (see Materials and Methods), after which the stained protein spots were cut, digested, and counted by LSC (see Materials and Methods).

From the electropherograms of ribosomes labelled with 0.1 mM [^{3}H]IAP, (Figure 18, A and C, absence and presence of CAP, respectively), the ribosomal proteins can be seen to be well resolved, especially L2 (arrow). The fluorograms of the dried gels (Figure 18, B and D, absence and presence of CAP, respectively) show L2 as the most intensely labelled protein (Figure 18, B, arrow) and the amount of label is greatly reduced on L2 (Figure 18, D, arrow) when CAP, the affinity labelling blocker, is present.

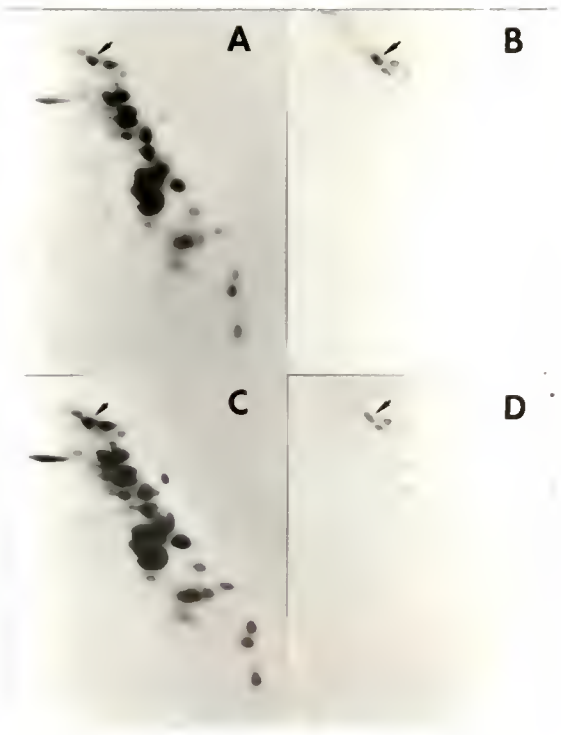


Figure 18. Two-dimensional PAGE analysis of the [^{3}H]iodoamphenicol ([^{3}H]IAP) labelling of mitochondrial ribosomal large subunit proteins, where chloramphenicol (CAP) was absent or present in the labelling reaction. Mitochondrial 39S subunits were incubated for one hour at 34°C with 0.1 mM [^{3}H]IAP in the absence (A and B) or presence (C and D) of 4.0 mM CAP. The ribosomal proteins were extracted, separated by 2-D PAGE, and analyzed by fluorography. From the electropherograms, A and C (absence and presence of CAP, respectively), a good resolution of the proteins is observed, especially in the region of L2 (arrow). The fluorograms (14 day exposure) reveal L2 (arrow) as the most intensely labelled protein (B) and that the label is extensively reduced in the presence of CAP (D), which competes with [^{3}H]IAP for the CAP binding site.

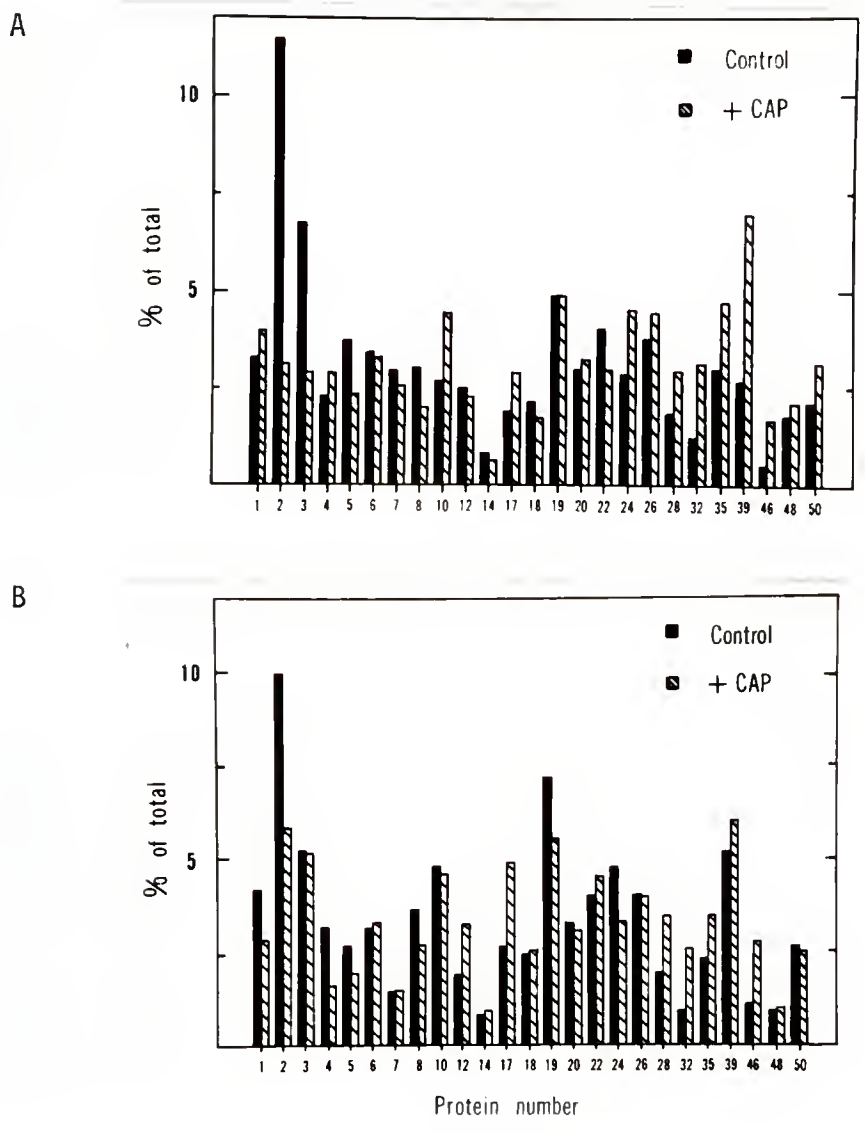


Figure 19. Comparison of label incorporated into individual proteins separated by 2-D PAGE, from mitochondrial ribosomal large subunits labelled by [^{3}H]iodoamphenicol ([^{3}H]IAP) in the absence and presence of CAP. Mitochondrial 39S subunits were incubated with 0.01 mM (A) or 0.1 mM (B) for one hour at 34°C in the absence and presence of 4.0 mM CAP. The ribosomal proteins were extracted, separated by 2-D PAGE, and analyzed by fluorography. The protein spots were cut, digested, and counted by liquid scintillation counting in parallel from the dried gels. The results are presented as a percentage of the total label from a given gel. From the histogram, L2 is seen to incorporate the most label of the proteins (filled boxes) and exhibits the greatest reduction of labelling in the presence of CAP (slashed boxes). Other proteins appear to exhibit an effect, but to a much lesser extent. Absence of CAP, filled boxes and presence of CAP, slashed boxes.

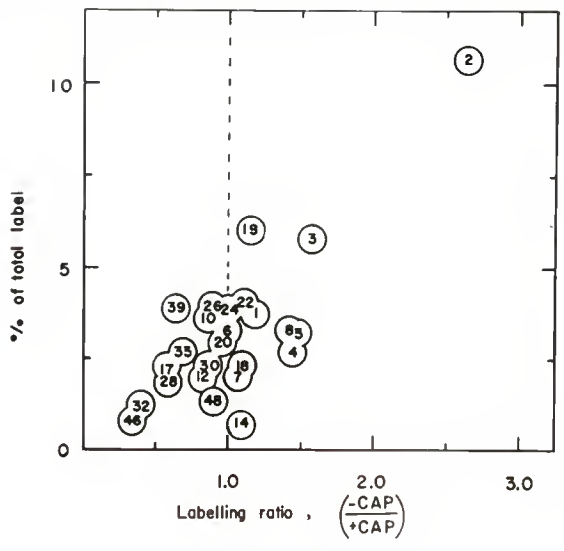


Figure 20. Comparison of the ratio of [^{3}H]iodoamphenicol ([^{3}H]IAP) incorporated into mitochondrial ribosomal large subunit proteins in the absence and presence of chloramphenicol (CAP) to the level of [^{3}H]IAP incorporated into the proteins. The data presented in Figure 19 was averaged and then the "labelling ratio" was calculated by dividing the amount of label observed in the absence of CAP by the amount of label observed in the presence of CAP. The amount of label in the absence of CAP is plotted on the ordinate versus its labelling ratio on the abscissa. Notice that most of the proteins are found in a zone about the labelling ratio of one (0.4 to 1.6), representing a lack of effect on the labelling by CAP. In contrast, L2 resides in a unique position of good labelling by [^{3}H]IAP with an extensive competitive effect on the labelling by the presence of 4.0 mM CAP.

The histograms of the ribosomal protein labelling pattern of 0.01 mM [^{3}H]IAP and 0.1 mM [^{3}H]IAP in the presence and absence of CAP show the same results, L2 is the most prominently labelled protein and CAP can prevent the affinity labelling of L2 by [^{3}H]IAP (Figure 19). A "scattergram" of the results of the [^{3}H]IAP affinity labelling of ribosomal proteins was constructed using the total amount of label appearing on a protein as one axis and the ratio of the labelling of a protein in the absence and presence of CAP as the other axis (Figure 20). It is apparent from the scattergram that L2 resides in a unique position of prominent labelling with an extensive reduction in labelling by the competitive effect of CAP, where the rest of the ribosomal proteins cluster about the ratio of one which represents equal labelling by [^{3}H]IAP whether the affinity labelling blocker, CAP, is present or is not present (Figure 20).

From the data presented about the specific affinity labelling of L2 by [^{3}H]IAP, it can be concluded that L2 is within the PTase Center of mammalian mitochondrial ribosomes and that L2 is a ribosomal protein at or near the CAP binding site of these ribosomes.

2-D PAGE analyses of [^{3}H]iodoamphenicol labelling of ribosomal proteins in the monosome

The mitochondrial large ribosomal subunit (39S) houses the CAP binding site and PTase Center and, in addition, [^{3}H]IAP specifically labels L2 from these sites on the

39S subunit (34). But the ribosome *in vivo* is a 55S monosome comprised of a large subunit and a small subunit (28S) (2, 3, 7). In considering the affinity labelling of ribosomal proteins of the 55S monosome, it was important to determine if the presence of the 28S subunit could change or interfere with the [^{3}H]IAP labelling of ribosomal proteins from the 39S subunit.

The mitochondrial ribosomal 55S monosomes were incubated with [^{3}H]IAP (0.1 and 1.0 mM) in the absence and presence of CAP (4.0 mM) (see Materials and Methods). The labelled monosomes were dissociated into subunits by a high salt, low Mg^{++} buffer and the subunits were separated on a linear (10-30%) sucrose density gradient (see Materials and Methods). The fractions containing the individual ribosomal subunits were pooled and centrifuged at 100,000 xg to concentrate the subunits (see Materials and Methods). The ribosomal pellets were solubilized into the reaction buffer and an A_{260} measurement was made, after which equal amounts of ribosomes were removed from each sample for ribosomal protein extraction (see Materials and Methods). The extracted ribosomal proteins were separated by 2-D PAGE after which the gels were analyzed by fluorography (see Materials and Methods).

There are several interesting features observable from the fluorograms . First, it should be noticed that [^{3}H]IAP fails to label L2, L10, L19 and L39, in the monosome (Figure 21), while these four proteins are among

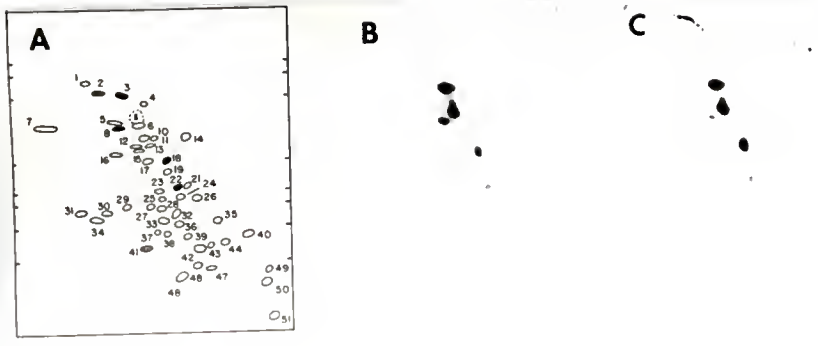


Figure 21. Two-dimensional PAGE analysis of the [^{3}H]iodoamphenicol ([^{3}H]IAP) labelling of mitochondrial ribosomal large subunit proteins labelled in the monosome in the absence and presence of chloramphenicol (CAP). ^{3}55S mitochondrial ribosomes were incubated with 1.0 mM [^{3}H]IAP in the absence (B) and presence (C) of 4.0 mM CAP for one hour at 34°C. The ribosomal subunits were separated on sucrose density gradients and the 39S and 28S peaks were pooled. The ribosomal proteins were extracted from the 39S subunits, separated by 2-D PAGE, and analyzed by fluorography (109 day exposure). A schematic of the gel is given in panel A where the most intensely labelled proteins are L3, L8, L18, and L22 (filled spots) and where a minor amount of label was observed on L2 and L41 (stippled spots). The heavily labelled spot, X, does not migrate to a position recognized as a large subunit protein, but it is coincident with the position of a heavily labelled small subunit protein, S4. Notice that there is no apparent labelling difference in the absence (B) or presence (C) of CAP.

the most intensely labelled proteins when [^{3}H]IAP is incubated with the 39S subunit (Figures 18, 19, and 20). Also, it is interesting to note that the affinity labelling blocker, CAP, has no apparent effect on the [^{3}H]IAP labelling of ribosomal proteins on either subunit in the 55S monosome, under these conditions (Figures 21 and 22). From the monosome labelling by [^{3}H]IAP, L3, L8, L18, and L22 are the most prominently labelled proteins, with L2 and L41 as minor labelled proteins from the large subunit (Figure 21). And S4 and S9 are the major labelled proteins with S2, S16, S24, and S34 as the minor labelled proteins from the small subunit (Figure 22).

L3 is the most intensely [^{3}H]IAP labelled protein from the monosome labelling reaction (the spot was visible after one week of fluorography) and L3 also labels well, but not "specifically" (approximately equal labelling whether CAP was present or not), with [^{3}H]IAP under the 39S labelling reaction conditions (Figure 20). L3 appears to contain a very reactive nucleophile (to be discussed later), so that it reacts well, but not with specificity towards the electrophilic affinity probe.

From the lack of a CAP effect on the [^{3}H]IAP labelling of ribosomal proteins, and the overall different labelling patterns of ribosomal proteins from the monosome as compared with the subunit labelling, it appears as though [^{3}H]IAP was behaving as a topological probe, the surface accessible proteins being the ones that label well.

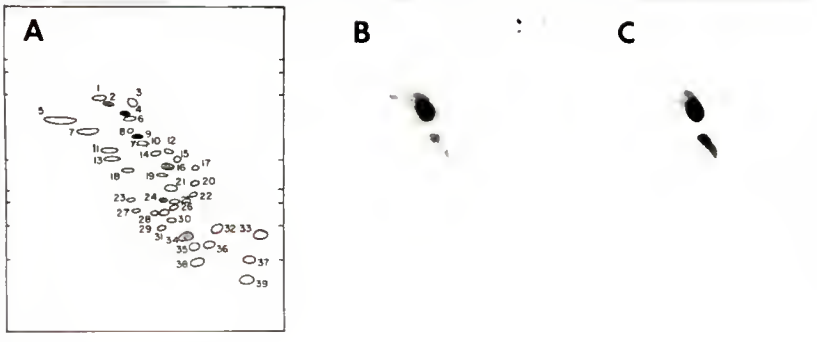


Figure 22. Two-dimensional PAGE analysis of the [^{3}H]iodoamphenicol ([^{3}H]IAP) labelling of mitochondrial small subunit proteins labelled in the monosome in the absence and presence of chloramphenicol (CAP). The labelled 28S subunit proteins were prepared as described in Figure 21. In the schematic diagram of the small subunit (A), S4 and S9 (filled spots) are shown to be labelled well and S2, S16, S24, and S34 (stippled spots) are shown as containing less label. There is no small subunit protein assigned to Y, as it appears to be where L18 migrates in the 2-D PAGE system employed. As with the large subunit, there appears to be no real [^{3}H]IAP labelling difference on these proteins when comparing their ability to label in the absence (B) or presence (C) of CAP. (Fluorograms exposed for 109 days)

Mitochondrial ribosomes have been studied by dual label iodine and dual label iodoacetic acid to determine the topological distribution of the ribosomal proteins, where the small subunit was found to be capable of masking the labelling of several proteins from the large subunit (Nancy Denslow, personal communication). This possibility suggests that L2, L10, L19 and L39 are proteins that are within the interfacial region of the ribosomal subunits so that the nucleophilic groups on these proteins are obscured when the subunits are in the monosome arrangement and thus are not available to label. Also, since L3, L8, L18, and L22 label well with [^{3}H]IAP in the monosome, these proteins would represent ribosomal proteins that surface at places other than the interfacial region. These descriptions for the ribosomal proteins labelled by [^{3}H]IAP in the large subunit from the monosome correlate well with data obtained from topological studies (Nancy Denslow, personal communication).

Effects of N-acetyl-AA-tRNA and AA-tRNA on the [^{3}H]iodoamphenicol labelling of ribosomal large subunit proteins

The Peptidyl Transferase Center contains the binding sites for each of the substrates of this reaction center, where N-acetyl-AA-tRNA and AA-tRNA bind to the P site and A site, respectively. The CAP binding site lies within the binding site for AA-tRNA since it interferes with the binding of the 3' end of AA-tRNA (20, 26). N-Acetyl-

AA-tRNA and AA-tRNA were used to determine if their binding to the ribosome could affect the [^{3}H]IAP labelling of ribosomal proteins that may be within the domain of these binding sites.

Mitochondrial ribosomal large subunits were incubated with [^{3}H]IAP and with N-acetyl-AA-tRNA (50 μM) or AA-tRNA (50 μM) (see Materials and Methods). Upon termination of the labelling reaction, the ribosomal proteins were extracted and separated by 1-D PAGE. The polyacrylamide gels were analyzed by fluorography and the fluorograms were scanned in a recording densitometer (see Materials and Methods).

Four regions of the unidimensional SDS gels contain identifiable proteins: I contains L2, II contains L10, III contains L19, and IV contains L39 (Figure 23A stained gels). These proteins are the most intensely labelled proteins in corresponding zones of 2-D gels of [^{3}H]IAP labelled proteins (Figures 18, 19, and 20), facilitating their identification. These protein assignments were confirmed using information from an independent analysis of individual proteins conducted in our laboratory (Gretchen Schieber, personal communication).

Both of the tRNA substrates can prevent the [^{3}H]IAP labelling of L2 (region I), L10 (region II), and L39 (region (IV) (see Figure 23). The AA-tRNA mixture can prevent the [^{3}H]IAP labelling of L19 (region III), whereas the labelling was not blocked as well by N-acetyl-AA-tRNA

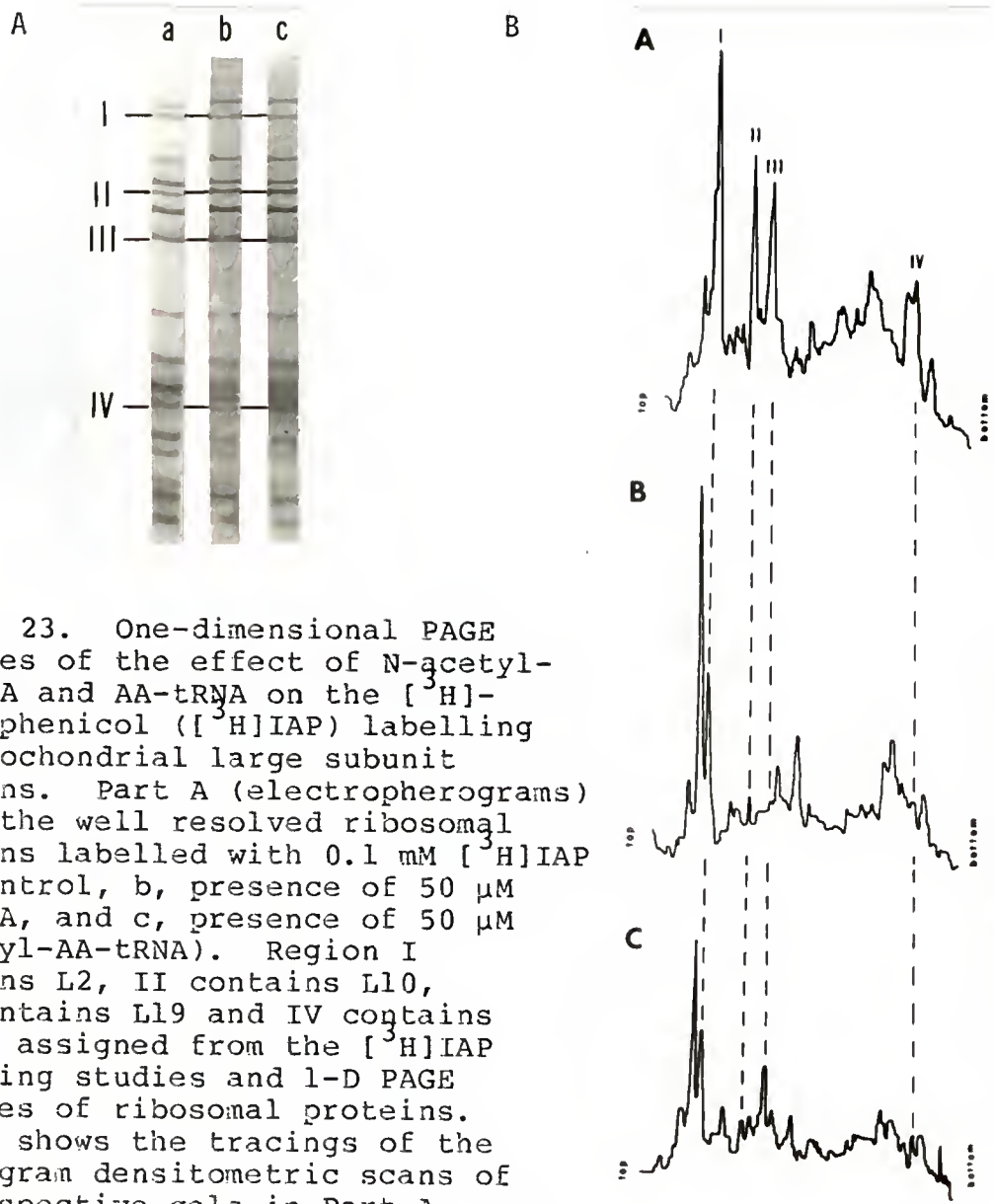


Figure 23. One-dimensional PAGE analyses of the effect of N-acetyl-AA-tRNA and AA-tRNA on the [^{3}H]-iodoamphenicol ([^{3}H]IAP) labelling of mitochondrial large subunit proteins. Part A (electropherograms) shows the well resolved ribosomal proteins labelled with 0.1 mM [^{3}H]IAP (a, control, b, presence of 50 µM AA-tRNA, and c, presence of 50 µM N-acetyl-AA-tRNA). Region I contains L2, II contains L10, III contains L19 and IV contains L39 as assigned from the [^{3}H]IAP labelling studies and 1-D PAGE analyses of ribosomal proteins. Part B shows the tracings of the fluorogram densitometric scans of the respective gels in Part A. The amount of label appearing in L2, L10, and L39 is reduced by both tRNA substrates (regions I, II, and IV, respectively) whereas the amount of label in L19 (region III) is reduced by AA-tRNA (B) but is not affected as much by N-acetyl-AA-tRNA (C). (Fluorograms exposed for 35 days)

(Figure 23B). Other regions of the tracings also show a differential labelling pattern by [^{3}H]IAP in the presence of the tRNA substrates, but the effect on these regions is not as dramatic. Ribosomal proteins in regions other than the four described above are not readily identifiable on 1-D polyacrylamide gels.

CAP exerts no effect on the [^{3}H]IAP labelling of L10, L19, and L39, so that these ribosomal proteins are not close enough to the CAP binding site to have their [^{3}H]IAP labelling affected by the binding of CAP. Yet the tRNA substrates appear to physically mask the [^{3}H]IAP labelling of these proteins. The tRNA substrates are approximately 75A long and span about one-third of the surface of the ribosomal large subunit. This comparatively large size allows the tRNA substrates to mask portions of the surface of the ribosomal large subunit in much the way that the ribosomal small subunit does (see the Monosome Labelling section). All four of these ribosomal proteins appear to be interfacial proteins, judging from the ability of the small subunit to prevent their labelling by [^{3}H]IAP, and from the masking of their labelling by the tRNA substrates (which bind to the ribosome at the interfacial region). From these data, it can be concluded that these four ribosomal proteins (L2, L10, L19, and L39) are exposed within a defined region on the surface of the ribosomal large subunit, that these

proteins are interfacially disposed, and they lie within the domain of the tRNA binding sites. The AA-tRNA used was possibly contaminated with some uncharged tRNA (which binds to the P site) so that when it was used to mask the ribosomal surface, both the A and the P site could be occupied. The N-acetyl-AA-tRNA should have only been bound to the P site, and this preparation of N-acetyl-AA-tRNA is not expected to contain any compounds which might bind to the A site. Therefore, L10 and L39 can be assigned to a region of the ribosomal interface within the P site. Since N-acetyl-AA-tRNA does not prevent the labelling of L19 very well, L19 can be assigned as being mainly in the region of the A site. The protein L2 appears to be located near the region of the P site (since N-acetyl-AA-tRNA blocks L2 labelling).

p-Nitroso-Chloramphenicol (NOCAP) as the Affinity Probe

In searching for other CAP analogs for use as affinity probes of the CAP binding site, NOCAP was selected because its structure is very similar to CAP (only one oxygen removed from CAP), the nitroso group is a reactive electrophile, and the nitroso moiety of NOCAP is distal to the iodoacetate (the reactive moiety) of IAP. It was thought that NOCAP might affinity label a different set of ribosomal proteins from those labelled by IAP, because it was a different electrophile and had the reactive moiety is in a different position on the CAP molecule.

Effect of p-nitroso-chloramphenicol on the peptidyl transferase activity of mitochondrial ribosomes

NOCAP was found to be an effective reversible inhibitor of the mitochondrial ribosomal PTase activity (Figure 4 and Table II). Therefore the CAP analog was tested as an irreversible inhibitor.

Mitochondrial ribosomal large subunits were incubated with 1.0 mM NOCAP, 1.0 mM CAP, or in the absence of antibiotic (control) for one hour at 34°C. The ribosomes were dialyzed overnight and centrifuged through a sucrose cushion to remove any non-covalently attached inhibitor. The ribosome samples were tested in the PTase assay to observe for any irreversible inhibition produced by NOCAP alkylation of the ribosomes. These conditions had been used previously to demonstrate the irreversible inactivaiton of the mitochondrial ribosomal PTase activity (Mary Conde, personal communication). Some of the ribosomes treated with NOCAP were also assayed for PTase activity in the presence of CAP, to determine if NOCAP covalently attached to the ribosome could occlude the CAP binding site, and thereby make the ribosomes insensitive to CAP. This phenomenon is observed with erythromycin and the bacterial ribosomes (25). Erythromycin does not inhibit the PTase activity of bacterial ribosomes, but it does interfere with CAP binding to these ribosomes; therefore bacterial ribosomes are insensitive to CAP in the presence of erythromycin (25).

TABLE IV

Peptidyl Transferase Activity of Mitochondrial Ribosomes After a Prolonged Treatment with Chloramphenicol or p-Nitroso-Chloramphenicol

Antibiotic Present During Incubation	Antibiotic Present During the Peptidyl Transferase Assay	Peptidyl Transferase Activities (CPM)
None	none	469*
CAP (1 mM)	none	495
NOCAP (1 mM)	none	552
NOCAP (1 mM)	CAP (40 μM)	240

*All values have units of counts per minute

Mitochondrial ribosomes were treated with the reagent indicated for one hour at 34°C, dialyzed overnight, and concentrated by centrifugation. The activities were determined using the peptidyl transferase assay which included the indicated conditions and are reported as the counts per minute minus the background observed for the assay performed in the absence of ribosomes.

It was found (Table IV) that NOCAP did not irreversibly inhibit the mitochondrial ribosomal PTase activity. Furthermore, it did not render the ribosomes insensitive to CAP, under the conditions employed (Table IV). Notice that an approximately 50% inhibitory level of CAP (40 μM) does reduce the activity of NOCAP treated ribosomes by about one-half, and that ribosomes treated with NOCAP appear to have a somewhat higher activity than the untreated control ribosomes. This implies that if NOCAP is covalently attaching to ribosomal proteins, then none of the proteins have a nucleophile in a functionally important region of the PTase Center. Alternatively, a one hour incubation may not be long enough to allow sufficient labelling of functionally important proteins, for a significant irreversible inactivation of the PTase activity to be observed. Labelling studies with [^{14}C]NOCAP show low ribosomal protein reactivity with the probe in support of the latter statement.

1-D PAGE analysis of [^{14}C]NOCAP labelling of ribosomal large subunit proteins in the absence and presence of chloramphenicol

Even though NOCAP could not be shown to irreversibly inactivate the mitochondrial ribosomes, I felt that it was imperative to use NOCAP as an affinity probe for proteins of the CAP binding site. The failure to observe irreversible inactivation of the PTase activity could have been the result of too short of an incubation, so that there was insufficient NOCAP bound covalently to the CAP binding site,

or perhaps the CAP binding site is without a precisely oriented nucleophile to allow complete occlusion of the CAP binding site upon covalent reaction with NOCAP. (The low value of irreversible inactivation of the PTase activity by IAP tends to support the latter conclusion.) Since NOCAP could reversibly bind to the CAP binding site, there must be affinity direction toward nucleophiles near the CAP binding site even if there is not one precisely oriented within it.

[^{14}C]NOCAP was synthesized from [^{14}C]chloramphenicol which limited the amount of [^{14}C]NOCAP produced and therefore the number of labelling experiments which could be done (see Materials and Methods). [^{14}C]NOCAP was incubated with mitochondrial large subunits for one hour at 34°C using a concentration range of 5 μM to 0.5 mM. The ribosomal proteins were separated by unidimensional SDS PAGE and analyzed by fluorography (see Materials and Methods). Insufficient radioactivity was incorporated into the ribosomal proteins at the lower levels of [^{14}C]NOCAP.

The electropherograms of the stained gels (0.5 mM [^{14}C]NOCAP) are shown in Figure 24, A and C, (absence and presence of CAP, respectively), where the protein band numbering scheme is the same as used in the [^{3}H]IAP and [^{14}C]IAM labelling studies. From the fluorograms (B, absence of CAP and D, presence of CAP), it is apparent that several protein bands are differentially labelled in the

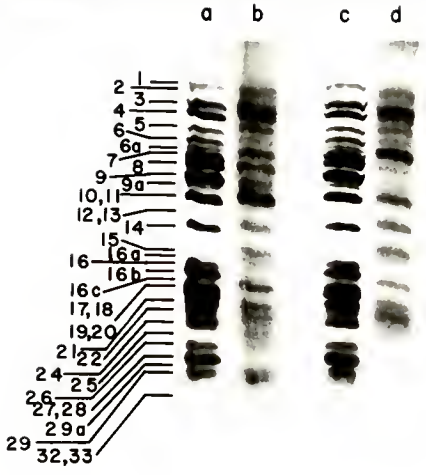


Figure 24. One-dimensional PAGE analysis of the affects of chloramphenicol (CAP) on the [^{14}C]nitroso-chloramphenicol ([^{14}C]NOCAP) labelling of mitochondrial ribosomal large subunit proteins. Mitochondrial 39S subunits were incubated with 0.5 mM [^{14}C]NOCAP in the absence (A and B) and presence (C and D) of 4 mM CAP for one hour at 34°C. The ribosomal proteins were extracted, separated by 1-D PAGE, and analyzed by fluorography. The electropherograms (A, absence of CAP and C, presence of CAP) show the well-resolved proteins in which the bands are numbered as in the [^{3}H]IAP and [^{14}C]IAM labelling studies. From inspection of the respective fluorograms (48 day exposure) (B, absence of CAP and D, presence of CAP), it is apparent that the presence of CAP exerts a reduction of labelling on several proteins, most notably, Bands 4, 8, 9, 10, 11 and 29.

presence of CAP. Bands 29, 8, 10, 11, and 4 (13,250 MW, 30,000 MW, 27,500 MW, 27,000 MW and 45,000 MW, respectively) exhibit the most prominent differential labelling effect (Figure 24).

The protein bands were cut, digested, and counted by LSC. The results are shown as a "scattergram" (Figure 25), where the extent of the label incorporation for each protein band is on the ordinate and the ratios of the label incorporated in the presence and absence of CAP are on the abscissa. Band 29 is found in a position by itself (Figure 25), one of high label incorporation and of a good CAP competition effect on the [^{14}C]NOCAP labelling. Although most of the protein bands exhibit no CAP effect, by having a ratio of about one, a few of the protein bands have an intermediate character which may represent some degree of affinity labelling. Among these, Bands 10, 11, and 4, which show a marginal effect, Band 9, which exhibits more of an effect, and Band 8 which exhibits an even greater effect, when CAP is used to compete with the [^{14}C]NOCAP in the labelling reaction. The labelled proteins in Bands 4, 8, and 10, 11 have been identified provisionally as L2, L10, and L19, respectively, by the [^{3}H]IAP labelling studies, where Band 9 is unassigned. Since this study used a different labelling reagent ([^{14}C]NOCAP as opposed to [^{3}H]IAP), these protein band assignments can only be considered tentative at the present time, as it cannot be presumed that [^{14}C]NOCAP will affinity label the same

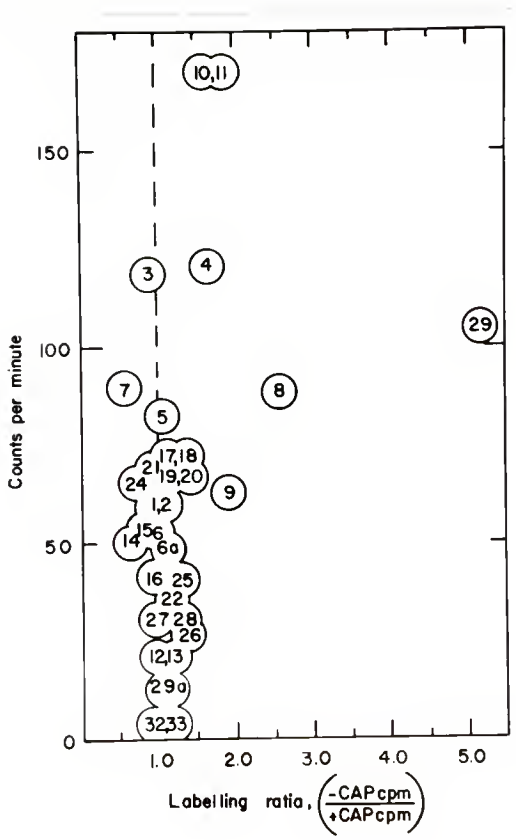


Figure 25. Comparison of the ratio of [^{14}C]nitroso-chloramphenicol ([^{14}C]NOCAP) incorporated into mitochondrial ribosomal large subunit proteins in the absence and presence of chloramphenicol (CAP) to the level of [^{14}C]NOCAP incorporated into the proteins. The protein bands, shown in Figure 24, were cut, digested, and counted by liquid scintillation counting to determine the amount of label present in the parallel gels. The ratio of the amount of label per band in the absence of CAP to the amount of label per band in the presence of CAP was calculated as a labelling ratio and appears as the abscissa in the plot. The ordinate is the amount of label per band in the absence of CAP. As had been observed in the [^{3}H]IAP labelling studies (Figure 20), most of the proteins cluster about the labelling ratio of 1.0 (0.5 to 1.5) indicating the lack of a CAP effect. A few protein Bands, 4, 9, and 10, 11, exhibit a marginal CAP effect whereas Band 8 shows a definite effect and Band 29 shows an extreme CAP competition effect.

proteins that [^{3}H]IAP does. Although the ribosomal protein in Band 29 has not been identified, it is interesting to note that L2, a high molecular weight CAP binding site protein, was identified by the [^{3}H]IAP affinity labelling studies and now a low molecular weight protein, Band 29, has been detected by [^{14}C]NOCAP affinity labelling. A similar situation was reported for the bacterial CAP binding site where the bacterial ribosomal proteins L2 and L27 were affinity labelled (61).

CHAPTER IV
DISCUSSION

Chloramphenicol is a clinically relevant antibiotic, which is used extensively as the drug of choice for the treatment of meningial infections (93). CAP is being used routinely at the Shands Teaching Hospital (Logan Brooks, M.D., infectious diseases intern, personal communication). CAP would probably be used to treat other than the life threatening infections, if it were not for the serious side effects of aplasia exhibited as an irreversible aplastic anemia (94). A reversible aplasia has been associated with the inhibition of protein synthesis within the mitochondria of bone marrow cells by CAP whereas the lesion for the irreversible aplastic anemia has not been identified (95, 96, 97, 98, 99, 100, 101). Nonetheless, the role of CAP in binding to the mitochondrial ribosomes is thought to be an important event in the processes leading to aplasia. A more thorough understanding of the manner in which CAP interacts with the mitochondrial ribosomes may bring about a way to circumvent this serious side-effect.

The binding of CAP to bacterial ribosomes, along with the components of the CAP binding site, have been fairly well studied (43). Yet, when I initiated these studies, little was known about the interaction of CAP with the

mitochondrial ribosomes, except that CAP could inhibit the PTase activity of these protein-rich particles (34, 85). I was interested in determining which of the ribosomal proteins were involved with the binding of CAP and if any of these proteins may be involved in the functions of the Peptidyl Transferase Center. Affinity labelling was chosen as the technique for identifying these proteins and IAP was chosen as the first affinity labelling reagent to be used on the mitochondrial ribosomes, since it had previously been used to identify the CAP binding site protein, L16, from bacterial ribosomes (59, 61).

In the present study, IAP was shown to be able to interact with the mitochondrial ribosomes in a functionally significant manner, by its ability to reversibly inhibit the PTase activity of these ribosomes. Previously, IAP had been shown to irreversibly inactivate the PTase activity of mitochondrial ribosomes (Mary Conde, personal communication). With this functional interaction established, IAP was synthesized in a radiolabelled form, to be used to affinity label proteins in the CAP binding site of mammalian mitochondrial ribosomes.

From the [^{3}H]IAP labelling studies of mitochondrial large subunit proteins analyzed by 1-D PAGE, it was observed that a protein, in Band 4, labelled in a saturating, stoichiometric manner. In contrast, the labelling of Band 4 by [^{14}C]IAM exhibits a different, non-saturating profile, similar to the other proteins labelled by

[^{14}C]IAM. Overall, the other protein bands exhibit a similar concentration-dependent labelling pattern whether [^{3}H]IAP or [^{14}C]IAM is the labelling reagent used and none appear to be labelling with a saturating profile.

Chloramphenicol was used to competitively block the [^{3}H]IAP labelling of ribosomal proteins as a specificity control. CAP exerted a competition of labelling effect only on the protein in Band 4. The differential labelling effect is probably not the result of a conformation change of the ribosomes in the presence of CAP, as was inferred from the lack of a CAP effect on the labelling of ribosomal proteins with [^{14}C]IAM. The different labelling patterns of Band 4 by [^{3}H]IAP (saturating at stoichiometry) and by [^{14}C]IAM (a non-specific labelling reagent, which labels in a non-saturating manner) taken together with the competitive blocking effect by CAP, provide good evidence for the specific affinity labelling of the protein in Band 4. By the same criteria, other proteins such as Bands 8, 10, 11, and 24 do not exhibit specific affinity labelling by [^{3}H]IAP.

Mitochondrial ribosomal protein L2 (45,000 MW) was readily identified as the affinity labelled protein in Band 4 from 2-D PAGE analyses of the [^{3}H]IAP labelling of proteins from the mitochondrial ribosomal large subunit. In addition, other proteins that label well with [^{3}H]IAP could be identified from the 2-D PAGE analyses and these were assigned to the bands already identified

from the 1-D PAGE analyses, where Band 8 is L10, Band 10, 11 is L19, and Band 24 is L39.

From these data, it was concluded that L2 was a protein in or near the CAP binding site and within the Peptidyl Transferase Center of mammalian mitochondrial ribosomes.

To obtain further information about the ribosomal proteins that label with [^{3}H]IAP, additional studies of the [^{3}H]IAP labelling of ribosomal proteins were conducted using the 55S monosome rather than the 39S large subunit as the probe target. It was observed that the presence of the 28S small subunit could mask the labelling of many of the proteins on the large subunit. From their respective fluorograms, the most intensely labelled proteins from the large subunit labelling studies with [^{3}H]IAP (L2, L10, L19, and L39) are only faintly labelled in the monosome labelling study. Another interesting observation was that the presence of CAP had no real effect on the labelling by [^{3}H]IAP of ribosomal proteins from either subunit in the monosome. L3 was the most intensely labelled protein in the monosome labelling studies, and since CAP does not block the labelling of L3 by [^{3}H]IAP, its extensive labelling may solely be the result of the presence of a powerful nucleophile on this protein.

All indications were that [^{3}H]IAP was behaving as a surface topography probe and that the interfacial proteins were being protected from labelling in the monosome labelling conditions. The mitochondrial ribosomes have been

topologically examined by dual label iodine studies and dual label iodoacetic acid studies. The results are essentially the same as observed from the masking of the [^{3}H]IAP labelling by the small subunit, that L2, L10, L19, and L39 are ribosomal proteins located in the interfacial region of the ribosomes (Nancy Denslow, personal communication) (102). In addition, functional studies of the CAP binding site of mitochondrial ribosomes have revealed that the small subunit may actually interfere with the binding of CAP to its binding site on the large subunit, which in effect could reduce or possibly obliterate any affinity labelling that would be directed from the CAP site (Nancy Denslow, Ph.D. dissertation).

In further studies, N-acetyl-AA-tRNA and AA-tRNA were used to affect the [^{3}H]IAP labelling of ribosomal large subunit proteins. The labelling differences observed in the presence of N-acetyl-AA-tRNA are the result of its binding to the P site, but the effect of AA-tRNA on the labelling patterns may be the result of A and P site binding, as a result of contamination with uncharged tRNA. N-Acetyl-AA-tRNA could prevent the [^{3}H]IAP labelling of L2, L10, L39, and to a much less extent, L19, where AA-tRNA prevents the labelling of all four proteins. Since L2 is the only one of these four proteins identified as being in or near the CAP binding site, the tRNA substrates appeared to be capable of physically masking these proteins when bound to the ribosomes and thereby prevent their labelling

by [^{3}H]IAP, in much the way that the small subunits were able to mask the ribosomal interfacial proteins.

In continuing the affinity labelling studies for proteins from the CAP binding site of mitochondrial ribosomes, another affinity probe, NOCAP, was selected for use. NOCAP is actually closer in chemical structure to CAP than IAP and NOCAP has a different reactive moiety placed in a different position of the molecule than the iodo moiety of IAP.

NOCAP was capable of reversibly inhibiting the mitochondrial ribosomes very well, but irreversible inactivation of the PTase activity was not observed under the reaction conditions employed. The lack of irreversible inactivation might have been the result of an insufficient time during the incubation with NOCAP so that not enough of the CAP binding sites had covalently attached probes. Alternatively, a precisely oriented nucleophile may not be present in the site, to allow the covalently attached NOCAP to reside within the CAP binding site. Both of these are viable possibilities, since the [^{14}C]NOCAP labelling study showed a low reactivity of [^{14}C]NOCAP with ribosomal proteins and, when IAP was bound to the ribosomes stoichiometrically, only about 25% irreversible inactivation was observed. This latter possibility indicates that the reactive nucleophile may be slightly removed from the actual CAP binding site. Even though NOCAP did not irreversibly inactivate the ribosomal PTase activity, it could interact

in a reversible manner. Therefore, [^{14}C]NOCAP was synthesized and used to affinity label ribosomal large subunit proteins.

From the 1-D PAGE analysis of the [^{14}C]NOCAP labelling of mitochondrial ribosomes, only a few protein bands appear to exhibit a specific CAP competition of labelling effect. Bands 29 and 8 are affected to a great extent whereas Bands 4, 10, 11, and 9 exhibit only marginal effects. Proteins L10 for Band 8, L2 for Band 4, and L19 for Band 10, 11 have been identified in the [^{3}H]IAP labelling studies, but these assignments can be considered only tentative with the different probe, [^{14}C]NOCAP. Bands 29 and 9 are unassigned. If the [^{14}C]NOCAP labelled protein in Band 8 is L10, then this protein is probably near the actual CAP binding site.

From the data gathered in the affinity labelling studies, I propose a topological model of the 39S mitochondrial ribosomal interfacial region (Figure 26). Ribosomal protein L2 is assigned to the Peptidyl Transferase Center in the region where CAP (triangle) binds, since CAP can block the [^{3}H]IAP affinity labelling of this protein. L2 has also been identified as one of four possible PTase Center proteins from functional studies of the mitochondrial ribosomes, where the PTase activity was abolished after high-salt washes of the ribosomes with the concommitant loss of L2 and three other proteins (Robert Hessler, personal communication) (102). Near the CAP

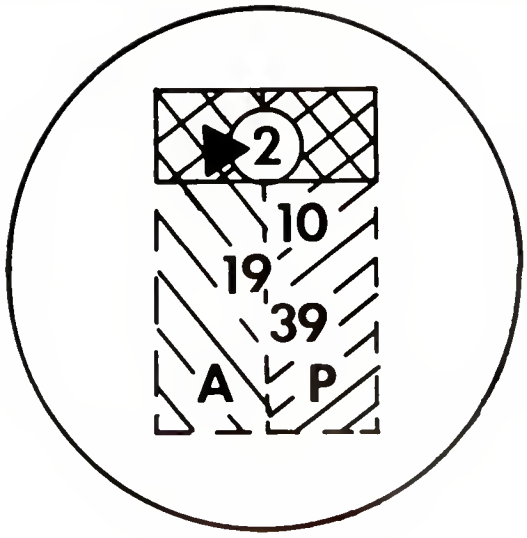


Figure 26. Model of the topological map of proteins from the interfacial region of the mitochondrial ribosomal large subunit. The model was constructed from the information about the proteins obtained from the affinity labelling studies. The circle represents the ribosome, with the interfacial region being viewed head-on. The A site is marked by the down-sloping hatched lines and the P site is marked by the up-sloping hatched lines. The Peptidyl Transferase Center is marked by the crosshatched lines and the chloramphenicol binding site is represented by the filled triangle. See the Discussion for the details of the placement of the proteins.

binding site and in the P site is ribosomal protein L10, since the tRNA substrates prevent its labelling by [^{3}H]IAP and because there is CAP blockage of a [^{14}C]NOCAP labelled protein tentatively assigned as L10. Ribosomal protein L19 is farther from the CAP binding site because no effect was observed on its labelling by [^{3}H]IAP in the presence of CAP, even though there may have been a marginal CAP effect of the [^{14}C]NOCAP labelling of a protein tentatively identified as L19. Furthermore, L19 is assigned to be mainly in the A site from the tRNA substrates effect on its labelling by [^{3}H]IAP. Ribosomal protein L39 is assigned to a position in the P site, from the studies where the tRNA substrates prevent its labelling by [^{3}H]IAP. L39 is positioned some distance from the CAP binding site, where CAP is not able to exert a significant effect on its labelling by [^{3}H]IAP.

Future refinements of the model will occur as new information about the positions and involvements of the ribosomal proteins in the Peptidyl Transferase Center and CAP binding site of mammalian mitochondrial ribosomes are discovered.

In the search for new and more efficient chloramphenicol affinity analogs, a prerequisite for the ability of the analogs to inhibit the ribosomal peptidyl transferase activity had to be established. After assaying several CAP analogs, there appeared to be patterns emerging

whereby the structural features of the analogs could be related to their functional activities.

The CAP analogs tested in my studies belong to two categories, p-nitro substituted analogs (ACAP, HACAP, NOCAP, FAP, and PAP) and dichloracetate substituted analogs (IAP and PAF). Some of the analogs contain photo-activatable moieties (FAP, PAP, and PAF) and others contain electrophilic moieties (IAP, NOCAP, and HACAP). Additionally, four of the analogs are a closely related family, by virtue of seemingly minor p-nitro substitutions (ACAP, HACAP, NOCAP, and PAP). Since the analogs have various structural interrelationships, their respective abilities to act as PTase inhibitors could be related structurally.

Of all the CAP analogs assayed, only PAF and PAP were found not to inhibit the PTase activities of mitochondrial and bacterial ribosomes. This was not so surprising for PAF, since the constraint of the size of the dichloro moiety had already been established for the bacterial ribosomes (36, 39, 89, 91). From the present study, I suggest that a similar size constraint probably also exists for the mitochondrial ribosomal CAP binding site, since IAP (with an iodoacetate substitution that does not exceed the size constraint of the dichloroacetate) is inhibitory and PAF (with a bulky phenylazido substituent) is not.

Yet, there was no previous indication that PAP would not be inhibitory. On the contrary, it had been reported in

many studies that the electronegativity of the para-nitro moiety was the feature of this position contributing to the inhibitory capacity of CAP (29, 36, 38, 39, 69, 70, 90). I found that PAP was inactive towards both bacterial and mitochondrial ribosomes, in spite of the high electronegativity of the azido moiety. PAP has been synthesized by others, but its use as a successful affinity probe has not been reported, probably due to its inability to bind to the ribosomes (64, 65, 88).

When the CAP reduction analogs were synthesized and assayed, I was surprised that ACAP had the ability to inhibit the ribosomal PTase activities. ACAP had been reported to be inactive (69, 87). Its activity had not been tested though, only speculated (87). Using the data from Garrett, I was able to calculate a K'_d which was identical to the one that I had obtained for ACAP from the PTase assays of bacterial ribosomes (69). There appears to be a semantic discrepancy in the literature, when terms such as "active" and "inactive" are used to describe CAP analogs, but are not defined as to their reference conditions. By using a single low concentration of the antibiotics in the assays, CAP may show activity, whereas a CAP analog may not. Yet at higher concentrations, the CAP analog may be very active. For these reasons, I have presented my data as inhibition curves so that the activity of the antibiotics can be observed over broad concentration ranges (see

Results), and thereby not be restricted to a narrow definition of activity.

NOCAP and HACAP were also found to be good inhibitors of the mitochondrial ribosomal and bacterial ribosomal PTase activities. This was not unexpected since their structures are very similar to the structure of CAP (Figure 2).

The results of the PTase assays of PAP, ACAP, NOCAP, and HACAP, provided data for analyzing the effects of the para substituents on the ability of the analogs to inhibit the PTase activities of the ribosomes, for a family of closely related analogs. Initially the electronegativity (Hammett sigma value) was used in the correlation, where a poor fit was observed for the analogs (71). The highly electronegative PAP was inactive and the poorly electronegative ACAP and HACAP were more active than predicted from their electronegativity. Since nitro groups are well known for the H-bonding ability, I selected this physical feature as a parameter that could be important for the binding of CAP to its binding site.

The ability of the analogs to inhibit the PTase activites of the ribosomes correlated well with the H-bond strengths of the p-nitro substituents. The inactive analog, PAP, has a poor H-bond forming group (72, 73, 92).

From these data, I propose that the essential feature of the p-nitro position of CAP is its ability to form a hydrogen bond to some, as yet, undefined component in the CAP binding site. This is not unreasonable, since H-bonds

are known to be important physical forces for the interaction of molecules, and in addition the hydroxyl moieties of CAP have previously been shown to H-bond to bacterial ribosomes (44).

Considering that for a CAP analog to be capable of binding to the CAP binding site, the presence of a hydrogen bond acceptor in the para position is required, FAP was proposed as a novel photo-activatable CAP analog, where the para-amido moiety would meet the requirement. Even with the extra bulk from the para position, FAP was observed to be inhibitory towards the PTase activities of bacterial and mitochondrial ribosomes over a meaningful concentration range. This implies that the carbonyl may serve as the H-bond acceptor. Also, the extra bulk from the para position did not inactivate this CAP analog, in accordance with previous reports about this position (29, 36).

The three-dimensional (3-D) structure of CAP has been determined using x-ray crystallography, NMR spectroscopy, and molecular orbital calculations (70, 103, 104). CAP is folded into a "V" shape, with the propanediol as the apex and the p-nitrophenyl ring and the dichloroacetate chain forming the trailing arms (103, 104). From studies on the energy of activation of CAP binding to bacterial ribosomes, it was determined that CAP does not undergo conformational changes upon binding (44). The binding of CAP to the ribosomes appears to follow the classical lock and key model, without dynamic changes in the substrate or binding

site (44). The mitochondrial ribosomes probably bind CAP and the CAP analogs in a similar manner.

From the data of the functional studies with CAP analogs, I propose a model for the CAP binding site applicable to both bacterial and mitochondrial ribosomes, in which the important features of CAP and the CAP binding site are highlighted (Figure 27). CAP is represented in its 3-D form, with the important points of interaction indicated (Figure 27). The dichloro moiety is within a pocket with an undefined mode of interaction (stippled zone) with the binding site. No pocket constraint is shown extending from the p-nitro position, since it has been reported that bulky substituents in the para position do not greatly interfere with the binding of CAP analogs (29, 36). The aromatic ring of CAP interacts with some ribosomal component (crosshatched zone), probably by hydrophobic interactions.

Three positions are represented as H-bond formers, X, Y, and Z, where X H-bonds to the p-nitro moiety and Y and Z H-bond to the hydroxyls of the propanediol chain.

From the present study, it appears as though the CAP binding site of mammalian mitochondrial ribosomes contains the same essential features as the one from bacterial ribosomes: 1) pocket for the dichloro moiety, 2) no pocket constraint from the para position, and 3) the newly proposed feature of hydrogen bonding for the p-nitro moiety.

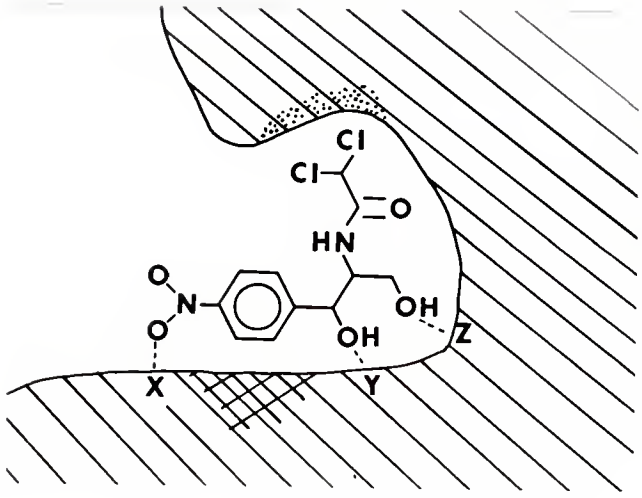


Figure 27. Detailed model of the chloramphenicol binding site. The model is proposed from information about the functional interaction of CAP and CAP analogs with the ribosomes and is applicable to both mitochondrial and bacterial ribosomes. The hatched region is the ribosomal surface. The crosshatched region represents an interaction with the aromatic ring. The stippled region represents an interaction with the dichloro moiety. X, Y, and Z represent ribosomal groups that hydrogen bond to the nitro moiety and propanediol chain. Chloramphenicol is shown in its three-dimensional conformation. Notice the pocket for the binding of the dichloro moiety and the lack of a pocket at the p-nitro moiety.

These features should be carefully considered in the design and synthesis of CAP analogs, to avoid a needless waste of time and resources.

In conjunction with the model presented about proteins near the CAP binding site, a more complete picture of the CAP binding site of mammalian mitochondrial ribosomes has emerged.

APPENDIX I

BUFFERS

Buffer A - 0.34 M sucrose, 5 mM Tris, pH 7.5

Buffer B - 0.25 M sucrose, 1 mM EDTA, 5 mM Tris, pH 7.5

Buffer C - 44.4 mM KCl, 16.6 mM $MgCl_2$, 2.2 mM 2ME,
4.4 mM Tris, pH 7.5, 0.11 mM spermine,
0.11 mM spermidine

Buffer D - 300 mM KCl, 40 mM $MgCl_2$, 5 mM 2ME,
1.6% Triton X-100, 10 mM Tris, pH 7.5

Buffer E - 100 mM KCl, 20 mM $MgCl_2$, 5 mM 2ME,
20 mM triethanolamine, pH 7.5

Buffer F - 100 mM KCl, 30 mM $MgCl_2$, 5 mM 2ME,
0.1 mM EDTA, 10 mM Tris, pH 7.5

Buffer G - 500 mM KCl, 10 mM $MgCl_2$, 5 mM 2ME,
10 mM Tris, PH 7.5

Buffer H - 25 mM KCl, 25 mM $MgCl_2$, 5 mM 2ME,
10 mM Tris, pH 7.5

Buffer I - 40 mM KCl, 10 mM $MgCl_2$, 6 mM 2ME,
10 mM Tris, pH 7.5

Buffer J - 500 mM KCl, 5 mM $MgCl_2$, 5 mM 2ME,
20 mM triethanolamine, pH 7.5

Buffer K - 444.4 mM KCl, 22.2 mM Mg Acetate,
1.1 mM puromycin, 55.6 mM Tris, pH 7.5

Buffer L - 200 mM KCl, 10 mM Mg Acetate,
25 mM triethanolamine, pH 7.5

Buffer M - 15.05% acrylamide, 0.55% bisacrylamide,
0.2% SDS, 730 mM Tris, pH 8.8,
0.05% tetraethylenediamine

Buffer N - 9.74% acrylamide, 0.26 bisacrylamide, 0.2% SDS,
375 mM Tris, pH 6.8, 0.67% tetraethylenediamine

Buffer O - 2% SDS, 5% 2ME, 50% glycerol, 62.5 mM Tris, pH 6.8

Buffer P - 0.1% SDS, 0.01% mercaptoacetic acid, 192 mM glycine, 25 mM Tris, pH 8.3

Buffer Q - 9 M urea, 0.01% aminoethanethiol, 60 mM potassium acetate, pH 6.7

Buffer R - 8 M urea, 4.5% acrylamide, 0.09% bisacrylamide, 60 mM potassium acetate, pH 4.3, 0.13% tetraethylenediamine

Buffer S - 8 M urea, 3.86% acrylamide, 0.14% bisacrylamide, 60 mM potassium acetate, pH 6.7, 0.055% tetraethylenediamine

Buffer T - 8 M urea, 4.54% acrylamide, 0.16% bisacrylamide, 60 mM potassium acetate, pH 6.7, 0.055% tetraethylenediamine

Buffer U - 0.01% aminoethanethiol, 35 mM Beta-alanine, pH 4.5

Buffer V - 5.1 M urea, 0.5% SDS, 9.65% acrylamide, 0.35% bisacrylamide, 100 mM sodium phosphate, pH 7.2, 0.1% tetraethylenediamine

Buffer W - 5.1 M urea, 0.5% SDS, 3.86% acrylamide, 0.14% bisacrylamide, 10 mM sodium phosphate, pH 7.2, 0.06% tetraethylenediamine 0.1% riboflavin

Buffer X - 0.5% SDS, 0.025% mercaptoacetic acid, 100 mM sodium phosphate, pH 7.2

APPENDIX II

RIBOSOMAL QUANTITATION BY UV SPECTROSCOPY

1.0 A_{260} = 55 pmole 39S

1.0 A_{260} = 83 pmole 28S

1.0 A_{260} = 32 pmole 55S

1.0 A_{260} = 23 pmole 70S

1.0 A_{260} = 33 pmole 50S

MOLECULAR WEIGHTS OF CHLORAMPHENICOL AND THE CHLORAMPHENICOL ANALOGS

CAP - 323 MW

ACAP - 293 MW

HACAP - 309 MW

NOCAP - 307 MW

PAP - 333 MW

FAP - 438 MW

PAF - 356 MW

APPENDIX III

PHOTOLYTIC STUDIES OF FAP

FAP is a reversible inhibitor of the PTase activites of bacterial and mitochondrial ribosomes, in contrast to the other photo-activatable CAP analogs, PAF and PAP (see Results). Photolytic experiments were begun to determine which conditions would be required to bring about photo-incorporation of FAP onto ribosomal proteins.

FAP was photolyzed in a quartz cuvette by a Hanovia 400 watt high pressure mercury vapor lamp at a distance of 10 cm from the source. The photolysis of FAP was monitored by measuring the absorption spectra of the solution at various times of irradiation. As observed in Figure 28, the absorbance at 280 nm decreases and there is a corresponding increase in absorbance at wavelengths greater than 310 nm during the photolytic period, with no further changes in absorbance after about ten minutes irradiation. The half-time of FAP photolysis, followed by the change in absorbance at 280 nm, was determined to be about 1.8 minutes (Figure 29).

An unfortunate problem with FAP is that the photolysis product is still inhibitory in the PTase assay

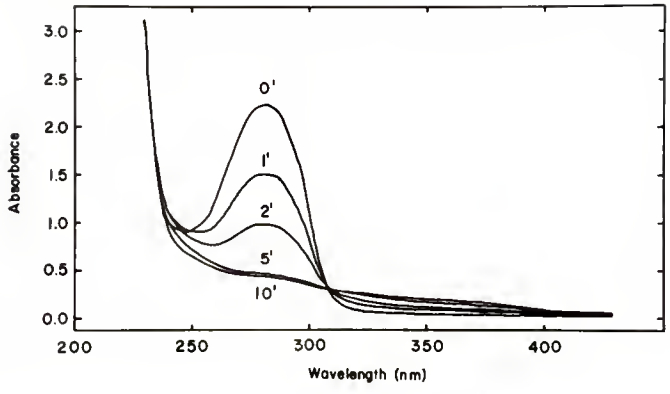


Figure 28. Spectra of the photolytic decay of p-azido-benzoamido-chloramphenicol (FAP). FAP was exposed to the UV light from a Hanovia 400 watt high pressure mercury lamp at a distance of 10 cm for one, two, five, and ten minutes. A spectrum was recorded at each time interval. Notice the decrease in absorbance at 280 nm with an increase in absorbace at wavelengths greater than 310 nm.

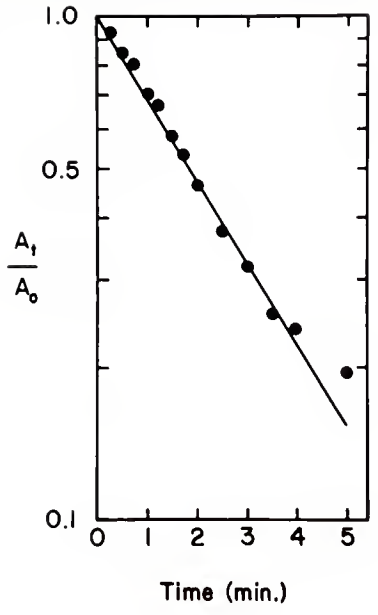


Figure 29. Determination of the half-time of the photolytic decay of p-azidobenzoamido-chloramphenicol (FAP). From the time course of the photolytic decay of FAP (as in Figure 28) a ratio of the change in absorbance at 280 nm was determined and plotted against the time. From the midpoint of the line, a half-time of 1.8 minutes was determined.

(data not shown), and therefore would act competitively to prevent the entry of unphotolyzed FAP into the CAP binding site during the affinity labelling photolysis. This and other problems inherent to photo-affinity labelling will be examined in the future.

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BIOGRAPHICAL SKETCH

Terry was born in Clermont, Florida, and grew up in the central Florida area, graduating as valedictorian of the Class of 1971 from Groveland High School.

He worked full-time as a mechanic in a local citrus plant while attending Lake-Sumter Community College. Terry finished his undergraduate studies at Florida Southern College, graduating magna cum laude with a B. A. in biology during the spring of 1976.

It was while attending Florida Southern College that Terry met his future wife, the former Jean Schmidt.

Since the fall of 1976 Terry has been a graduate student in the Department of Biochemistry and Molecular Biology at the University of Florida, with Professor Thomas O'Brien as his mentor.

Future plans include attending medical school and a career of bio-medical research.

Terry and Jean enjoy outdoor activities including nature photography, running, and especially bicycle touring.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Thomas W. O'Brien

Thomas W. O'Brien, Ph.D.
Professor and Acting Chairman
of Biochemistry and Molecular
Biology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Ben M. Dunn

Ben M. Dunn, Ph.D.
Associate Professor of
Biochemistry and Molecular
Biology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Peter M. McGuire

Peter M. McGuire, Ph.D.
Assistant Professor of
Biochemistry and Molecular
Biology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

David N. Silverman

David N. Silverman, Ph.D.
Professor of Pharmacology and Therapeutics and of Biochemistry and Molecular Biology

This dissertation was submitted to the Graduate Faculty of the College of Medicine and to the Graduate Council, and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

December, 1982

[signature] 11/03/82

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